(51) International Patent Classification 5 :		(11) International Publication Number:	WO 93/10461
G01N 33/573, C12Q 1/42 G01N 33/53	A1	(43) International Publication Date:	27 May 1993 (27.05.93)

(54) Title: NON-RADIOACTIVE ENZYME ASSAY

(57) Abstract

The present invention is directed to the assay and purification of proteins, and particularly to the non-radioactive assay and purification of protein kinases, phosphatases and protease by incubating the enzyme with a substrate modified peptide to form a product modified peptide under conditions where the enzyme is active. The product modified peptide and substrate modified peptide are then separated, and the product modified peptide is measured. The present invention is also directed to kits and bioreagents for performing the assays.

NON-RADIOACTIVE ENZYME ASSAY

FIELD OF THE INVENTION

The present invention relates generally to
prot in assays. More specifically, the invention relates
to assays of enzymes. The present invention relates
particularly to a non-radioactive method of assaying the
activity of protein kinases, phosphatases and proteases.

REFERENCE TO CITED ART

Reference is made to the section preceding the
CLAIMS for a full bibliography citation of the art cited
herein.

DESCRIPTION OF THE PRIOR ART

Enzymes are large proteins that catalyze
reactions in living cells. As used in this discussion,
the term "catalyze" refers to a substance that increases
the velocity of a reaction over the velocity of an
uncatalyzed reaction without itself undergoing a change.
Enzymes are specific in their reactions. An individual
enzyme may catalyze the reaction of one substrate or of a
group of related substrates.

In healthy persons, most enzymes are found
within cells. Some diseases, however, release enzymes
from dying cells into the blood. The increased levels of
enzymes can then be measured. An abnormal level of
enzymes in the blood characterizes certain medical
conditions. For example, an enzyme assay for abnormal
levels of creatine kinase in the blood is useful as a
diagnostic of heart disease. When a heart attack occurs,
cells are damaged and their enzymes leak into the
bloodstream. Enzyme levels increase markedly immediately
following a heart attack. Therefore, a confirmation of a
heart attack can be made by analyzing the enzyme levels
in the blood. In like manner, bone or liver diseases can
be diagnosed by increased levels of alkaline phosphatase
in the blood stream. Prostate cancer is diagnosed by
increased levels of acid phosphatase. Reference is made
to McMurry (1989) for a general description of the
medical uses of enzymes.

Enzymes are classified into groups according to the general kind of reaction they catalyze. For purposes of the present invention, reference is specifically made to two of the groups: (1) Transferases, which catalyze

5   the transfer of a group from one substrate to another; and (2) Hydrolases, which catalyze the hydrolysis, i.e., the breakdown of proteins to amino acids, of substrates. The present invention is specifically directed to the transferase subgroups kinases and phosphatases, and the

10 hydrolase subgroup proteases.

Kinases

Protein kinases are enzymes which covalently modify proteins and peptides by the attachment of a phosphate group to one or more sites on the protein.

15 Many of the known protein kinases use adenosine triphosphate (ATP) as the phosphate donor and place the gamma phosphate from this molecule onto a histidine (His or H), tyrosine (Tyr or Y), serine (Ser or S) or threonine (Thr or T) residue in the protein. The

20 location of the site of modification and the type of residue modified by the kinases is determined by the specific kinase under study.

Studies have shown kinases to be key regulators of many cell functions, including signal transduction

25 (Ullrich and Schlessinger, 1990), transcriptional regulation (Pawson and Bernstein, 1990), cell motility (Miglietta and Nelson, 1988), and cell division (Pines and Hunter, 1990). Several oncogenes, such as raf and src, have been shown to encode protein kinases (Bellas et

30 al., 1991), suggesting that kinases play a role in oncogenesis. Indeed few, if any, physiological processes exist in eukaryotes that are not dependent on phosphorylation. It has been postulated that the total number of protein kinases encoded in the mammalian genome

35 alone could number in the thousands (Hunter, 1987). Due to their physiological relevance, variety, and ubiquitousness, protein kinases have become one of the

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most important and widely studied family of proteins in biochemical and medical research.

Protein kinases are often divided into two groups based on the amino acid residue they phosphorylate. The Ser/Thr kinases include cyclic AMP-(cAMP-) and cGMP-dependent protein kinases, calcium- and phospholipid-dependent protein kinase C, calmodulin dependent protein kinases, casein kinases, cell division cycle (CDC) protein kinases, and others. These kinases are usually cytoplasmic or associated with the particulate fractions of cells, possibly by anchoring proteins (Carr et al., 1991).

The second group of kinases, which phosphorylate Tyr residues, are present in much smaller quantities, but play an equally important role in cell regulation. These kinases include several receptors for molecules such as growth factors and hormones, including epidermal growth factor receptor, insulin receptor, platelet-derived growth factor receptor, and others. Structure-function studies have indicated that many Tyr kinases are transmembrane proteins (Todderud and Carpenter, 1989), with their receptor domains located on the outside of the cell and their kinase domains on the inside.

Recent studies in yeast have proven the existence of a protein kinase that phosphorylate His residues on substrate proteins. The significance of this N-linked phosphorylation has yet to be explored (Matthews et al., 1991).

Phosphatases

Protein phosphatases are enzymes that catalyze the removal of phosphate moieties from proteins which contain such modifications. The sites which can be dephosphorylated is dependent upon the specific enzyme under study. Four major types of protein phosphatases have been identified, which have been grouped into two classes: type 1 protein phosphatase (protein

phosphatase-1 or PP1) and type 2 protein phosphatases
(PP2A, PP2B, and PP2C). The classes are distinguished by
their substrate specificity and dependence on other
molecules for activation.

As with kinases, both Ser/Thr and Tyr
phosphatases have been found. Peptide substrates for
individual protein phosphatases have been investigated
and identified (Kennelly and Krebs, 1991).

Proteases

Proteases have also been found to be important
in many biological processes. Some of these processes,
such as the formation of a fibrin clot, which begins the
healing process for wounds, involve many different
proteases acting in concert. When these proteases do not
act as they should, the results can be devastating. The
many forms of hemophilia are the result of mutations in
proteases. In performing research on the role of
proteases on cellular function, it is essential to
accurately and rapidly determine the amount of active
protease present in samples. For a general discussion on
these enzymes in such process, reference is made to
Neurath (1989), which reference is incorporated herein by
reference.

Activity Determination

Reference is made Robyt and White (1990) for a
general description of methods for determining the
activity of an enzyme. Robyt and White define the
activity of an enzyme as the amount of reaction that a
certain amount of enzyme will produce in a specified
period of time. The activity is determined by measuring
the amount of product produced or the amount of substrate
used up per unit of time under high concentrations or
saturating conditions of substrate. This is usually
accomplished by performing a chemical analysis for the
product or substrate.

Radioactive Detection of Activity

Most current methods of measuring protein kinase activity are based on the radioactive detection method described by Roskoski (1983). In this method, a sample containing the kinase of interest is incubated with activators and a substrate in the presence of gamma ^{32}P-ATP. Often, a general and inexpensive substrate, such as histone or casein, is used. After a suitable incubation period, the reaction is stopped and an aliquot of the reaction mixture is placed directly onto a filter which binds the substrate. The filter is then washed several times to remove excess radioactivity, and the amount of radiolabelled phosphate incorporated into the substrate is measured by scintillation counting (Roskoski, 1983). This method is widely used and provides an accurate method for determining protein kinase activity in both crude and purified samples.

The use of ^{32}P in the assay poses significant disadvantages. A major problem is the disposal of the radionuclide. For sensitive detection, relatively high quantities of ^{32}P must be used routinely and subsequently disposed. The amount of liquid generated from the washings is not small, and contains ^{32}P. Due to government restrictions, this waste cannot be disposed easily. It must be allowed to decay, usually for at least six months, before disposal. Another disadvantage is the hazard of working with the isotope. Shielding, special waste containers and caution are necessary for safe handling and pose great inconvenience and potential danger to workers. The half life of ^{32}P also provides a problem: with the half life of two weeks, the labelled ATP must be replaced often. Also, restrictions on the use of radioactivity, especially overseas, make the use of ^{32}P labelled ATP cumbersome. Further still, the lower detection limit of the assay is determined by the level of background phosphorylation and is therefore variable. In short, the study of protein kinases would be greatly facilitated by

the development of an efficient and accurate assay that does not require the use of radioactivity.

Although as ubiquitous and as important in cellular regulation as protein kinases, protein phosphatases are not studied as extensively. This is partially due to the difficulty in assaying the enzymes. Protein phosphatase assays contain the same difficulties as assays of protein kinases, with further complications. Assays of protein phosphatases require radioactively labelled phosphorylated substrates, which must be created specifically for this task. These substrates decay rapidly and are hazardous to work with. Most assays of phosphatase activity are measured by the removal of a label from the substrate. The increased difficulty in these assays is due to the fact that the substrate protein or peptide has a limited shelf life, and the isotope has a half life of two weeks. After labelling, the peptide is incubated with the phosphatase, and the resulting decrease in labelled peptide is measured by conventional means.

A non-radioactive method to measure protein phosphatase activity, perhaps using stable phosphorylated peptide substrates, would greatly aid research in this area. By providing non-radioactive substrates that can be easily quantitated after dephosphorylation, the researcher avoids the difficulties inherent with using ^{32}P; the non-radioactive substrates are also stable for long periods of time, avoiding the problems involved with radioactive decay.

Non-Radioactive Detection of Activity

Dot Blot

A non-radioactive method of detecting kinase activity has been developed in which tyrosine phosphorylation is detected by using anti-phosphotyrosine antibodies (Rijksen et al., 1989). After incubation of the tyrosine kinase with unlabelled ATP and a suitable substrate, the reaction mixture is subjected to a dot

blot assay on a PVDF membrane. The extent of phosphorylation is determined by reaction with anti phosphotyrosine antibody, followed by detection with an immunogold staining procedure. The amount of

5   phosphotyrosine present is detected with a densitometer. Aside from the absence of radioactivity, this method has the advantage of low background. Because phosphoserine is not detected, the sensitivity of the detection is increased.

10   The main disadvantage of the dot blot method is that it is limited to detecting Tyr kinases. Antibodies to phosphotyrosine can be produced due to the size of the antigen. Attempts to produce similar antibodies to phosphoserine and phosphothreonine have not been

15   successful. In addition, the assay requires several incubation and washing steps, each of a considerable length, which results in a long assay time. Finally, the result of the assay is a colored dot on the blot which limits the effective sample range of the assay and

20   requires that the user quantitate the final result by use of a scanning densitometer, a piece of equipment not available in all laboratories. The densitometer must have beam dimensions covering at least the targeted cross section of the dots generated.

25   Spectrophotometric Assay

Use of a modified substrate allows spectrophotometric detection of kinase activity. In this application, the cAMP-dependent protein kinase substrate Kemptide (Bramson et al., 1980) is modified by placing a

30   (o-NO$_2$)-tyrosine reside on the N-terminal side of the phosphorylated serine. This modification does not interfere with the ability of the peptide to be a substrate, and causes phosphorylation to alter the absorbance of the peptide at 430 nm. This allows

35   continual measurement of kinase activity, whereas measurement by radionuclide requires removal of an

aliquot of the reaction before ph sphate incorporation could be measured.

Unfortunately, the spectrophotometric assay requires large amounts of substrate as well as kinase to
5   determine activity effectively. While useful for mechanistic studies requiring a continuous measurement of phosphorylation, this method may not be useful when limited amounts of kinase are available or when the researcher must screen a large number of samples for
10 relative kinase activity. In addition, specific peptides are needed for every protein kinase and it is not known if (1) such modifications will be tolerated by all kinases and (2) if these changes will alter the specificity of phosphorylation for a peptide by a
15 particular kinase.

Other spectrophotometric assays for kinase activity have been developed using coupled reactions. The conversion of phosphoenolpyruvate to pyruvate can occur in the presence of ADP generated by kinase
20 phosphotransfer and pyruvate kinase (Cook et al., 1982). The pyruvate is then converted to lactate by lactate dehydrogenase and detected by reading the absorbance at 340nm. While effective, the method does have disadvantages: in crude samples, any ATPase activity
25 will increase the background and the intrinsic oxidoreductive reactions present in such extracts will interfere with activity measurements. In addition, the assay is not very sensitive.

Fluorescence Assay

30 Another non-radioactive detection method for kinase activity involves a change in fluorescence upon substrate phosphorylation. The substrate Malantide, a tetradecapeptide derived from the phosphorylation side of the ß-subunit of phosphorylase kinase, is an effective
35 substrate for cAMP-dependent protein kinase, and exhibits a decrease in fluorescence upon phosphorylation. Like the spectrophotometric assay, it allows continual

measurement of kinase activity. However, it uses large
amounts of synthetically prepared peptide, and it
requires access to a fluorometer.

5   Another type of assay is useful for cAMP
dependent protein kinase by use of a fluorescently
labeled peptide. The measurement of activity depends
upon the separation of the non-phosphorylated and
phosphorylated forms of the peptide by use of high
pressure liquid chromatography (HPLC) (Seng et al.,
10   1991). While this assay does not require the use of
radioactivity, it is cumbersome in that each sample has
to be individually injected and analyzed, thus requiring
more time for the assay of several samples than would be
possible using standard radioactive assays. Again,
15   quantitation of the products of the assay is performed
using fluorescent measurement of the amount of peptide
altered. Fluorometers are not commonly available in many
laboratories. Additionally, the assay may not be
functional in crude enzyme preparations. Because most of
20   the assays measuring protein kinases are performed on
crude samples and because it is not known if the peptide
would be degraded in crude peptide mixtures, the ultimate
utility of this assay is questionable.

Protease Assays

25   Proteases are mainly assayed in two different
ways: by digestion of specific substrates which allow
the researcher to directly monitor the activity of the
enzyme or, by digestion of a relatively nonspecific
substrate which can allow the activity of general
30   proteolytic activity to be measured. For a general
treatise on protease assays, reference is made to Sarath
et al. (1989), which is incorporated herein by reference.
          Proteases have also been assayed by cleavage of
large proteins such as casein and hemoglobin. When such
35   assays are performed, either the amount of hydrolysis is
followed by measuring the release of free protons
generated by the new carb xyl group formed in the

reaction or by measurement of the amount of trichloroacetic acid (TCA) soluble peptide generated from the substrate using a variety of reagents such as Folins reagent (Sigma Chemical Corp., St. Louis MO.). In

5 addition, some large substrates such as azocasine have been chemically modified to produce a substrate molecule with colored dyes attached. Proteases can be assayed using such substrates by measurement of the TCA soluble peptide generated by digestion of the substrate by the

10 protease. Such assays are undesirable as they do not distinguish between different proteolytic activities. Also the solubility of such substrates, coupled with the low detectability of the dyes bound to the substrate, often results in an assay with very poor sensitivity.

15 An additional protease assay has been reported which utilizes a substrate which has several fluorescent tags attached to one substrate. The fluorescent tags quench the emission of light from the return of any one tag to the ground state due to the close proximity of the

20 dyes in space. Proteolytic cleavage of the substrate by proteases allows the segments of the peptide carrying these labels to diffuse apart from each other, thus increasing the fluorescence of the solution carrying the substrate and allowing the activity of the protease to be

25 measured. This type of assay, however, requires that the substrate be soluble when it contains several such tags and these tags have been known to cause insolubility of peptides containing such modifications. In addition, such an assay will not be helpful in defining the

30 cleavage site within the peptide--thus allowing determination of the specificity of the protease--for any cleavage will generate the same signal.

If the technology currently used to study protein kinases, phosphatases and proteases could be

35 improved, knowledge of their function in metabolism would be increased. One technique used to study almost all areas of research involves the accurate and sensitive

detection of the enzyme activity. Assays for specific
kinases, phosphatases and proteases are used in many
applications, from purification to mass screening of
potential inhibitors. Improvements in the sensitivity,
5   selectivity, and rapidity of the assays would greatly aid
in their study.

SUMMARY OF THE INVENTION

The present invention is directed to a non-
radioactive enzyme assay which overcomes the deficiencies
10   discussed above. The assay can be performed rapidly and
at great sensitivity in both purified systems and in
crude samples of the enzyme. The method allows the assay
to be performed using commonly available equipment to
yield accurate results. The assay is generally directed
15   to the detection of protein kinases, phosphatases and
proteases.

The invention is specifically directed to a
non-radioactive method of quantitating the activities of
specific protein kinases, phosphatases and proteases on a
20   peptide substrate by measuring the amount of the specific
peptide substrate that has been reacted by the enzyme
under assay. Preferred steps include incubating the
enzyme with the substrate modified peptide to form a
mixture of substrate modified peptide and product
25   modified peptide under conditions where the enzyme is
active. The substrate modified peptide and product
modified peptide are then separated. The amount of the
product modified peptide that has been formed by the
enzyme under assay can then be measured.

30   The present invention is also directed towards
the assay of enzymes using substrate modified peptides
described in the invention. The use of these peptides
are advantageous in that they not only allow
determination of the activity of the protease, but also
35   can be helpful in confirming the specificity and purity
of the enzymes by generating sets of unique fragments
from the substrate modified peptide which are

characteristic of the type of proteolytic activity present in the sample.

The present is also directed to a bioreagent for quantitating the presence or activity of an enzyme, such as protein kinase, phosphatase or protease. The bioreagent is selected from the following group of peptides: Promega Peptide 1 (SEQ ID NO:1), Promega Peptide 2 (SEQ ID NO:2), Promega Peptide 3 (SEQ ID NO:3), Promega Peptide 4 (SEQ ID NO:4), Promega Peptide 5 (SEQ ID NO:5), Promega Peptide 6 (SEQ ID NO:6), Promega Peptide 7 (SEQ ID NO:7), Promega Peptide 8 (SEQ ID NO:8), Promega Peptide 9 (SEQ ID NO:9), Promega Peptide 10 (SEQ ID NO:10), Promega Peptide 11 (SEQ ID NO:11) and analogs thereof.

The invention is also directed to kits for quantitating the activities of an enzyme selected from the group consisting of protein kinases and protein phosphatases or proteases using a substrate modified peptide. The kits include at least a substrate modified peptide modified by chemical reaction to allow easy visualization/ quantitation of the substrate modified peptide and separation protocols for isolation of either the substrate modified peptide or product modified peptide.

The present invention is also directed towards a kit for performing protease assays, comprising a substrate or substrates for protease digestion made in accordance with the present invention and instructions on how to use the substrate for the assay of protease activity and preferably including protocols for the confirmation of the specificity of the protease under assay.

Surprisingly, a wide variety of peptide molecules which have been modified to allow their easy quantitation by a variety of means can be used as substrates for protein kinases, proteases and phosphatases. The measurem nt of the activity of th se

enzymes can be performed by separation of the product
modified peptide and substrate modified peptide species
and quantitation of the amount of either the residual
substrate modified peptide of the reaction, the product
5   modified peptide of the reaction, or both.

The enzymes present in a sample of a body
fluid, such as blood or lymph, can be measured within a
very short period of time with high accuracy by the assay
method of the present invention. The method includes
10   incubating a sample of the body fluid containing at least
one of the enzymes with the substrate modified peptide of
the present invention under conditions where the enzyme
will form a product modified peptide. By the use of the
substrate modified peptide, the enzyme can easily be
15   diagnosed in the body fluids and the appropriate disease
can be diagnosed.

The results are unexpected for at least three
reasons: (1) the enzymes have strict sequence
requirements, and therefore might not be thought to
20   recognize a peptide which has been modified chemically as
a substrate; (2) the modification tags used, while known
in the literature as species which allow quantitation of
very small amounts of a modified chemical species, often
result in the production of a modified species which has
25   a very low solubility in solvents needed for activity of
the enzymes under study; and (3) chemical species with
polyaromatic, hydrophobic structures, as disclosed in the
examples to follow, have been used to bind enzymes with
nucleotide substrates such as ATP. Such binding inhibits
30   the activity of the enzymes by preventing the entry of
the substrate molecules, thus preventing assay of the
enzymes in the presence of dyes.

Additionally, it was not known if the modified
peptides of the invention could withstand degradation in
35   complex samples, such as total cell lysates, where
various proteases and other enzymes might destroy the

peptides too rapidly to allow assay of other enzymes such as that of the protein kinases and phosphatases present.

In both pure and crude samples, the products of the invention have other unexpected advantages including the ability to assay the activity of an enzyme without measurement of other contaminating reactions which take place in the sample. For example, when protein kinases are measured in crude sample preparations using radioactive phosphate incorporated into added substrate and assay conditions well-known in the art, the activity of the assay actually measures the reaction of the enzyme with the substrate and the reaction of other kinases in the sample with protein and peptide substrates present in the sample. The present invention allows the measurement of only the modification of the specific peptide substrate added to the sample, thus avoiding the measurement of other activities in the sample.

Finally, an additional unexpected advantage to the system is its flexibility, which allows several options for sample separation including electrophoresis and chromatography.

BRIEF DESCRIPTION OF THE DRAWINGS

In the drawings:

Fig. 1 is a photograph illustrating the separation of phosphorylated Promega Peptides 1 (SEQ ID NO:1) and 2 (SEQ ID NO:2) from the unphosphorylated peptides by thin layer chromatography in Example 4.

Fig. 2 is a photograph illustrating the separation of phosphorylated Promega Peptides 1 (SEQ ID NO:1) and 2 (SEQ ID NO:2) from the unphosphorylated peptides by electrophoresis in Example 5.

Fig. 3 is a photograph illustrating the detection of small amounts of protein kinase using Promega Peptide 1 (SEQ ID NO:1) in Example 6.

Fig. 4 is a photograph illustrating the detection of protein kinase activity in crude extracts using Promega Peptide 1 (SEQ ID NO:1) in Example 7.

Fig. 5 is a photograph illustrating the synthesis of Promega Peptide 3 (SEQ ID NO:3) (Sulforhodamine 101 derivative of V^6A^5 Kemptide) in Example 8.

5   Fig. 6 is a photograph illustrating the purification of Promega Peptide 3 (SEQ ID NO:3) in Example 9.

Fig. 7 is a photograph illustrating an assay of protein kinase using Promega Peptide 3 (SEQ ID NO:3) in

10   Example 10.

Fig. 8 is a photograph illustrating the synthesis of Promega Peptide 4 (SEQ ID NO:4) in Example 11.

Fig. 9 is a photograph illustrating the

15   synthesis of Promega Peptide 5 (SEQ ID NO:5) in Example 11.

Fig. 10 is a photograph illustrating the synthesis of Promega Peptide 6 (SEQ ID NO:6) in Example 11.

20   Fig. 11 is a photograph illustrating the detection of protein kinase C activity using Promega Peptides 4 (SEQ ID NO:4), 5 (SEQ ID NO:5), and 6 (SEQ ID NO:6) in Example 13.

Fig. 12 is a photograph illustrating the

25   detection of protein kinase C activity in crude samples using Promega Peptide 6 (SEQ ID NO:6) in Example 14.

Fig. 13 is a photograph of an agarose gel used to analyze peptide species produced in the digestion of Promega Peptide 6 (SEQ ID NO:6) by modified trypsin and

30   Endoprotease Lys C in Example 16.

Fig. 14 is a photograph of an agarose gel used to analyze peptide species produced in the digestion of Promega Peptide 5 (SEQ ID NO:5) and Promega Peptide 7 (SEQ ID NO:7) by modified trypsin and Endoprotease Lys C

35   in Example 16.

Fig. 15 is a photograph illustrating assays of proteases in crude media samples in Example 17.

Fig. 16 is a photograph illustrating the quantitation of cAMP-dependent protein kinase activity using a spectrophotometer in Example 20.

Fig. 17 is a drawing illustrating an agarose gel photograph of the synthesis and purification of a tyrosine kinase modified peptide substrate, Promega Peptide 8 (SEQ ID NO:8) in Example 22.

Fig. 18 is a photograph illustrating the detection of alkaline phosphatase using a non-radioactive assay in Example 23.

Fig. 19 is a photograph illustrating the synthesis and purification of Promega Peptide 11 (SEQ ID NO:11) in Example 24.

Fig. 20 is a photograph illustrating the detection of protein kinase C activity using Promega Peptide 11 (SEQ ID NO:11) in Example 25.

DETAILED DESCRIPTION OF THE INVENTION

The present invention quantitates the activities of enzymes by measuring the amount of a specific modified peptide substrate that has been reacted by the enzyme under assay. Although the present invention is preferably directed to quantitating the enzymes protein kinase, phosphatase and protease, the invention has wider application. The present invention is contemplated to be used to assay phosphatases, kinases, proteases, methylases, de-methylases, glycosidases, and de-glycosidases and other enzymes.

For example, a coupled, modified peptide substrate having N-linked glycosidation on an Asn residue could be used to measure the activity of a de-glycosidase that removed N-linked glycosidation. The reaction would uncover a site for Endoprotease Asn N, which would otherwise be masked by the glucose residues.

In another example, a linked, modified peptide substrate having a Lys residue, which can be methylated by the methylases present in many cells, would mask this site for cleavage by prot ases such as Endoprotease Lys-

C. The activity of the enzymes which removes methyl residues from Lys residues can be measured by simply attaching a methylated peptide and measuring the amount of cleavable peptide that is generated.

5   <u>Definitions</u>

For purposes of the present invention, the following definitions will apply:

Activity of an Enzyme: The amount of reaction that a certain amount of enzyme will produce in a specified

10   period of time.

Amino Acids: Amino acids are shown either by three letter or one letter abbreviations as follows:

<u>Abbreviated Designation</u>		<u>Amino Acid</u>
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

Complex Sample: A sample consisting of more than 10

35   individual protein components.

Crude Sample: An extract from an entire organism or tissue made by lysis of the tissue or organism under mild conditions and removal of particular material.

Modified peptide: A chemical species composed of a

5 peptide as described below which has been subsequently reacted with a second chemical moiety which allows the peptide to be monitored by virtue of the properties of the second chemical moiety. This second chemical moiety will be referred to in this application as a detector

10 segment or modification tag. It should be noted that such a modified peptide can consist in almost all cases of other chemical elements in addition to amino acids, such as those modifications described in the definition of peptides.

15 Peptide: A compound consisting of 2-30 naturally occurring or synthetic amino acids which can also be further modified, as described above, which is covalently linked through peptide bonds formed from the α-carboxyl group of one amino acid and the α-amino group

20 of the next amino acid by elimination of a molecule of water. The amino acids can be either those naturally occurring amino acids described above, chemically synthesized variants of such amino acids such as norleucine, or modified forms of these amino acids which

25 can be altered from their basic chemical structure by addition of other chemical groups which can be found to be covalently attached to them in naturally occurring compounds. Some of the modifications so attached include: phosphate groups, lipid groups, nucleotide

30 groups, and polymers of sugars and will include other modifications known to those skilled in the art.

Phosphorylate: The addition of a phosphate group to a substrate.

Product modified peptide: A chemical species which

35 is formed by the action of an enzyme on a substrate modified peptide, as described below, which has been altered from the original bonding pattern between the

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atoms of the substrate modified peptide. Such alterations can include the addition or removal of new chemical species to the substrate modified peptide. The particular changes made in the substrate modified peptide

5 will depend upon the particular enzyme which has been involved in the alteration of the substrate modified peptide. Examples of such product modified peptides include but are not intended to be limited to: (a) a product modified peptide which had been formed by

10 incubation of a modified peptide with a protein kinase under conditions where the enzyme has altered the substrate modified peptide by addition of a phosphate group to the peptide from a phosphate donor such as ATP; (b) a product modified peptide which had been formed by

15 incubation of a substrate modified peptide with a protein phosphatase under conditions where the enzyme has altered the substrate modified peptide by removal of a phosphate group from the peptide; and (c) a product modified peptide formed by incubation of a substrate modified

20 peptide with a protease under conditions where the protease breaks one or more of the peptide bonds in the substrate modified peptide, thus producing chemical species where the detector segment is present on a chemical species with a smaller molecular mass than that

25 of the original peptide. It should be noted that the product modified peptide may be used as a substrate modified peptide is subsequent reactions; for example, a product modified peptide isolated from the incubation of a protein kinase with a substrate modified peptide which

30 acquired a phosphate group covalently attached to the peptide may be used by as a substrate modified peptide for measurement of the activity of a protein phosphatase in a subsequent reaction.

Pure Sample: A sample of less than 10 proteins.

35 Quantitation: The ability to numerically determine a property.

Sequence requirements: The property of an enzyme to have the ability to recognize and catalyze a reaction on only a subset of peptides due to the peptide sequence of these substrates.

5 Substrate: The substance on which an enzyme acts.

Substrate modified peptide: A modified peptide which can be changed by the action of an enzyme such that an alteration in the bonding pattern between the atoms of the modified peptide takes place. Such changes can

10 include the addition or removal of chemical species to the modified peptide. Often it will be desirable to design a particular substrate modified peptide for assay of a particular enzyme of interest. In such cases, it will be important to design such a substrate modified

15 peptide such that it contains the recognition sequence of the enzyme within its structure; such as including a lysine or arginine not followed by a proline in a substrate designed for assay of the protease trypsin. A potential substrate modified peptide contains the

20 particular site and form of an amino acid necessary for its functioning. For example, a potential substrate modified peptide for a protein phosphatase will have to contain a phosphorylated amino acid somewhere in its structure. A potential substrate modified peptide for a

25 protein kinase will have to contain an amino acid which can act as a phosphate acceptor such as a serine. The utility of a potential substrate modified peptide for assay of a particular enzymatic activity can be determined by incubating the potential substrate modified

30 peptide with the enzyme under conditions where the enzyme is known to be active and observing the rate with which product modified peptide is generated. In general, substrate modified peptides which can be converted to product modified peptides more rapidly will be considered

35 better peptides for assay of the enzyme.

Enzymes

          The present invention relates generally to the assay and purification of proteins, and specifically to enzymes, particularly to those proteins which modify proteins. The enzymes specifically described in this specification are kinases, phosphatases and proteases.

          The enzymes measured by the assay techniques of the present invention have strict sequence requirements. As such, they might not be thought to recognize a peptide which has been modified chemically as a substrate.

          A general description of the enzymes kinases, phosphatases and proteases is found in the prior art section of this patent application and is incorporated herein to describe the characteristics of these enzymes.

          Kinases - The kinases quantified in the present study preferably use adenosine triphosphate (ATP) as the phosphate donor and place the gamma phosphate from this molecule onto a His, Tyr, Ser or Thr residue in the protein. Protein kinases are distinguished by their ability to phosphorylate substrates on discrete sequences. These sequences have been determined by sequencing the amino acids around the phosphorylation sites, and are usually distinct for each protein kinase (Kemp and Pearson, 1990). Some overlap in targets have been observed with certain kinases. It has been shown that several different kinases can phosphorylate a common protein on different sites, such as is seen with glycogen synthetase (Roach, 1990). This recognition sequence on the substrate gives the different kinases great specificity and allows them to control different aspects of regulatory pathways.

          The substrate binding site on the kinase is believed to exist in the enzymes' catalytic domain. This domain is common to all protein kinases. It typically contains over 240 residues, and also contains the kinase's ATP binding site.

Phosphatases - Phosphatases derive from a group of enzymes known as hydrolases which hydrolytically cleave bonds with the consumption of water. Examples of phosphatases include alkaline and acidic phosphatases

5 which hydrolyse phosphoric acid esters.

Proteases - Proteases are able to cleave the peptide bond linking the carbonyl group of one amino acid with the amino group of the following amino acid. Several proteases are also capable of cleaving esters of

10 amino acids, releasing the hydroxy moiety as one product and the acid group as a second product.

The specificity of many proteases is defined by the amino acid or amino acids on the amino terminal side of the site of cleavage of the peptide chain. If the

15 amino acids on the amino side of the cleavage site is numbered as aa-1, aa-2, aa-3, etc. as they become farther removed from the cleavage site and aa+1, aa+2, aa+3 as they are found towards the carboxy terminal site of the cleavage site, any cleavage site can be defined by the

20 amino acids present in these locations. Schematically, the identification process can be presented as shown below:

Amino terminal   *cleavage site   carboxy terminal
to the site   to the site

25
```
        O           O           O           O           O
        ‖           ‖           ‖           ‖           ‖
...[AA-3]-C-N-[AA-2]-C-N-[AA-1]-C*-N-[AA+1]-C-N-[AA+2]-C-N-[AA+3]-C...
          |           |           |           |           |
          H           H           H           H           H
```
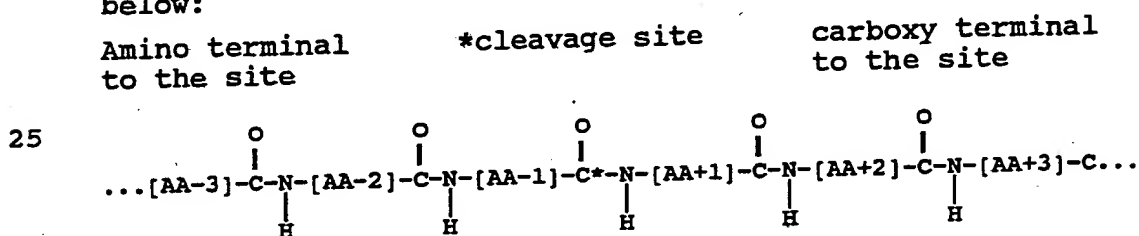
30   Using this system, the specificity of proteases can be defined by the range of sites that can be hydrolyzed by the enzyme. For example, trypsin will cleave a site when the aa-1 amino acid is Lys or Arg but only if the aa+1 amino acid is not a Pro residue. Factor

35 X is a much more specific protease and requires the sequence Arg - [AA + 1]. Although a number of amino acids can occupy the [AA + 1] position and allow cleavage by the enzyme, Ile and Thr are preferred and the enzyme

works slowly or not at all if the aa+1 amino acid is a
Pro residue.

Substrate

A wide variety of peptide molecules which can
be modified to allow their easy quantitation by a variety
of means can be used as substrates for kinases,
phosphatases and proteases. Preferred enzyme substrates
are those which will be modified by only a single kinase,
phosphatase or protease and have between 4-30 amino acid
residues.

The peptide substrates of the present invention
are molecules having the following characteristics:
(1) a substrate for one or more protein kinase,
phosphatase or protease; (2) a substrate labelled with a
detector segment which allows the presence of the peptide
to be measured, i.e., a substrate modified peptide; and
(3) a substrate which can be separated from the product
of the reaction by a variety of procedures including
electrophoresis, chromatography, and extraction as well
as others.

Substrates for Kinases - Several endogenous and
exogenous substrates have been described for different
protein kinases. In vitro, a single kinase can be shown
to have over a dozen substrates (House et al., 1987).
The mechanism by which a kinase can effectively
distinguish between substrates in vivo is thought to
involve compartmentalization and further regulatory
mechanisms. Although activation of kinases often leads
to profound physiological effects, the actual direct
effect of substrate phosphorylation is only beginning to
be understood.

Several common types of proteins, such as
histones and caseins, have been shown to be good
substrates for several protein kinases. This generality,
though, often limits the effectiveness of kinase
detection using such substrates by promoting a high
background of phosphorylation by interfering kinases.

Studies on kinase substrates have led to the determination of the amino acid sequences necessary on a substrate for phosphorylation. This has allowed the synthesis of peptide substrates that are highly specific

5 for individual kinase.

One of the best known artificial substrates is the heptapeptide Kemptide (Bramson et al., 1980), with the sequence:

L-R-R-A-S-L-G.

10 It is phosphorylated exclusively by the cAMP dependent protein kinase. Other specific substrates have been developed for other kinases, including cGMP-dependent protein kinase, calmodulin-dependent protein kinase (Pearson et al., 1985), and several Tyr kinases (Bowen et

15 al., 1990).

Substrates for Phosphatases - Substrates for protein phosphatases can be derived from the products of the reactions of various protein kinases (reference made to prior section) with the substrate peptides.

20 Substrates for Proteases - If specific substrates are designed for monitoring the activity of particular proteases, the specificity of the protease must be kept in mind. For example, substrates for trypsin are made with an Arg or Lys residue as the aa-1

25 so that the enzyme will recognize the substrate. Trypsin can also cleave esters of Lys or Arg, and if an ester of p-nitro phenol is made to Lys or Arg, the enzyme can release p-nitro phenol as a product of hydrolysis.

The activity of the enzyme can then be measured

30 by monitoring the amount of this product that has been produced, as the material absorbs light around 420nm when present as a free hydroxy group but absorbs much less light as an ester.

However, some proteases will not cleave such

35 esters--preventing their assay by such methods. In addition, other proteases cleave within their recognition site and will not release a colored ester the same way

that trypsin will; for the cleavage occurs between two
existing amino acids and not beyond the end of the
peptide which defines a substrate. Thus, while easy to
perform, these types of assays cannot be used with all
5   proteases.

Preferred substrates for the protease Factor X
have a Gly residue at the [AA - 2] position. A well-
known sequence, which is cleaved by this enzyme, is
represented by the following formula:

10   Ile-Glu-Gly-Arg * (Ile or Thr)

where the * indicates the site of cleavage (Owen et al.,
1974). Other proteases such as the HIV proteases have
been less well studied, but peptide substrates for these
proteases have been identified - sometimes through
15   analysis of the cleavage sites of the enzyme within
natural substrates. For example, the HIV protease has
been shown to cleave the following peptides:

R - S - L - N - Y * P - Q - S - K - W

20

A - T - L - N - F * P - I - S - P - W

where the * indicates the site of cleavage of the peptide
by the protease.

Detector Segment

25   The substrate is labeled with a detector
segment in a manner known to the art. The detector
segment allows the amount of the peptide to be measured.
The peptide detector segment, also referred to herein as
a detection sequence, chemical tag or modification tag,
30   is a chemical segment which allows the presence of the
peptide to be detected by virtue of the ability of the
chemical segment to absorb electromagnetic radiation such
as visible light, or by virtue of its ability to
fluoresce, i.e., emit light of a different wavelength
35   than it absorbs, or both.

The peptide detector segment which is present
on the peptide, now referred to as "substrate modified

peptide," can be of several types commercially available and commonly known to those skilled in the art. Examples include dansyl, sulforhodamine, fluorescein, rhodamine B (Lissamine Rhodamine) or other tags which allow the

5   measurement of the peptide to be performed.

Specialized Peptide Substrates

A number of specialized peptide substrates have been developed, each of which is tagged by a detector element as described above. Reference is made to Table 1

10   as follows for a listing of preferred proprietary peptide substrates:

Table 1

Peptide	SEQ ID NO:	Sequence	Modification (detection tag)
Promega Peptide 1	1	*-L-R-R-A-S-L-G	Dansyl
Promega Peptide 2	2	*-L-R-R-A-S-V-A	Dansyl
Promega Peptide 3	3	*-L-R-R-A-S-V-A	Sulforhodamine 101
Promega Peptide 4	4	*-R-F-A-R-K-G-S-L-R-Q-K-N-V	Lissamine Rhodamine#
Promega Peptide 5	5	*-Q-R-R-Q-R-K-S-R-R-T-I	Lissamine Rhodamine
Promega Peptide 6	6	*-P-L-S-R-T-L-S-V-A-A-K-K	Lissamine Rhodamine
Promega Peptide 7	7	*-L-R-R-A-S-L-G	Lissamine Rhodamine
Promega Peptide 8	8	*-D-R-V-Y-I-H-P-F	Lissamine Rhodamine
Promega Peptide 9	9	*-R-S-L-N-Y-P-Q-S-U-W	Lissamine Rhodamine
Promega Peptide 10	10	*-A-T-L-N-F-P-I-S-P-W	Lissamine Rhodamine
Promega Peptide 11	11	*-P-L-S-R-T-L-S-V-A-A-K	Lissamine Rhodamine

* Location of Detection Tag

Rhodamine B

A description of the formation, purification
and representative uses of Promega Peptides 1 - 11 (SEQ
ID NOS:1-11) follow in the examples.

Referring to Table 2, it has been determined
5   that the following Promega Peptide substrates have
specific utility for the named enzyme assay:

Table 2

Enzyme	Promega Peptide Substrates
Kinases (in general)	Promega Peptides 1-8, 11*
Tyrosine Kinase	Promega Peptide 8
Protein Kinase C	Promega Peptides 4,5,6,11
Protease	Promega Peptide 1-11
HIV Protease	Promega Peptide 9-10
Phosphatase	Modified (Phosphorylated) Promega Peptides 1-8, 11

*Refer to Table 1 for SEQ ID NO: listings

Measurement of Activity

Activity of an enzyme is determined by
measuring the amount of product produced, i.e., the
product modified peptide, or the amount of substrate
unmodified by the action of the enzyme per unit of time,
i.e., the substrate modified peptide. The measurement of
the activity of the enzymes can be performed by
separation of the modified and unmodified peptide species
and quantitation of the amount of either the residual
substrate of the reaction, the product of the reaction,
or both. The activity of protein kinases and
phosphatases can be measured because of their ability to
modify the specific peptide substrates described above by
addition or removal of modified or unmodified phosphate
groups from the peptide substrates of the invention.

The enzyme is incubated under conditions known
to the art with the substrate modified peptide to form
product modified peptides under conditions where the
enzyme is active. Each of the enzymes is usually assayed
at its optimum pH and temperature.

The enzyme may be incubated in a pure protein
sample, a complex protein sample or a crude sample, all
of which are defined above.

The detection of the activity of the enzyme is
performed by separation of the modified and unmodified
forms of the peptide in either qualitative or
quantitative measurement of the amount of both species
present in the sample.

The product modified peptide and substrate
modified peptide are separated from the product of the
reaction by a number of methods known to the art, such as
electrophoresis, chromatography and extraction.

As used herein, chromatography includes the
separation of materials by the movement of a solvent over
a separation matrix. Such chromatography can be
performed upon a silica gel matrix, a paper matrix, a
derivatized material used for ionic, hydrophobic, or size

exclusion separation of materials such as DEAE sephadex, Phenyl Sepharose, G25 Sephadex as well as other matrices well known to the art. Solvents for the separation will include aqueous, organic, non-organic, and mixtures of

5 aqueous and organic and non-organic solvents.

The amount of the specific product modified peptide that has been modified by the enzyme under assay is then measured. The activity of the enzyme is detected by measurement of the modified and unmodified forms of

10 the peptide. The measurement may be either qualitative or quantitative as defined below.

Quantitative Measurement

Spectroscopy. Quantitation can be performed using a spectrometer if the particular peptide used has

15 been designed to allow its measurement by absorption of electromagnetic radiation, such as visible or ultraviolet light.

Fluorescence. Quantitation can also be performed by the use of a fluorometer, if the particular

20 peptide used as a substrate can emit light of a particular wavelength after absorbing light of a different wavelength.

Chemiluminescence. Additionally, other types of quantitation, such as measurement of the production of

25 light from a particular peptide substrate modified to produce light by chemiluminescence, can be performed.

The present invention is also directed to kits that utilize the process of the present invention. A basic kit for quantitating the presence or activity of an

30 enzyme includes a container containing a bioreagent, which is a substrate modified peptide having specific reactivity to the enzyme, modified by chemical reaction to allow easy visualization/quantitation, and instructions for use.

35 The substrate modified peptide in the kit is preferably one of following peptides, depending upon the type of enzyme to be assayed as explained above: Promega

Peptide 1 (SEQ ID NO:1), Promega Peptide 2 (SEQ ID NO:2),
Promega Peptide 3 (SEQ ID NO:3), Promega Peptide 4 (SEQ
ID NO:4), Promega Peptide 5 (SEQ ID NO:5), Promega
Peptide 6 (SEQ ID NO:6), Promega Peptide 7 (SEQ ID NO:7),

5   Promega Peptide 8 (SEQ ID NO:8), Promega Peptide 9 (SEQ
ID NO:9), Promega Peptide 10 (SEQ ID NO:10), and Promega
Peptide 11 (SEQ ID NO:11). Reference is made to Table 2
above for substrate modified peptides specific for
certain enzyme assays.

10   The kit could further include a container
containing at least one buffer, which is compatible with
the enzyme. The kit can also include a container
containing at least one separation material for isolation
of either a product modified peptide or the substrate

15   modified peptide.

The present invention is also directed to a kit
for quantitating the activity of the enzyme protease in a
peptide substrate. The basic kit includes at least one
container containing at least one substrate respectively

20   for protease digestion, and instructions for use.

The amounts of the various reagents in the kits
can be varied depending on a number of factors, such as
the optimum sensitivity of the assay. The instructions
for use are suitable to enable an analyst to carry out

25   the desired assay. It is within the scope of this
invention to provide manual test kits or test kits for
use in automated analyzers.

The following examples are presented to
illustrate the advantages of the present invention and to

30   assist one of ordinary skill in making and using the
same. The examples are not intended in any way to
otherwise limit the scope of the disclosure or protection
granted by the patent.

While precise descriptions are given for assay

35   of protein kinases, phosphatases, and proteases in the
following examples, it should be clear to one well versed
in the art that the results of the present invention can

be applied to the assay of a wide variety of enzymes including but not limited to: esterases, deesterases, amidases, deamidases, glycosidases, deglycosidases, ribosylases, deribosylases, methylases and demethylases either directly or with minor modifications such as would be expected to one familiar with the assay of such enzymes.

While the material presented below takes advantage of peptides which are chemically synthesized _in vitro_ and some of which are commercially available, it should be noted that the invention can be performed by isolation of a peptide from a natural source by digestion of a larger precursor protein or peptide and isolation of the proper resulting fragment by methods well known in the art followed by its attachment to a detector segment as defined earlier.

EXAMPLES

Example 1

Synthesis of Promega Peptide 1 (SEQ ID NO:1)
(Dansyl Kemptide) and Promega Peptide 2 (SEQ ID NO:2)
(V^6A^5 Dansyl Kemptide)

A solution of Kemptide (Sigma Chemical Co., St. Louis, MO.) and V^6A^5 Kemptide (Sigma Chemical Co., St. Louis, MO.) was made by dissolving 5 milligrams (mg) of the peptide in 1 milliliter (ml) of 200 millimolar (mM) sodium borate buffer, pH 9.0. The solutions were placed into individual 1.5ml Eppindorf tubes. A control solution of the buffer without peptide was also prepared by placing 1ml of the buffer in a separate 1.5ml Eppindorf tube. To each of these solutions was added 50 microliters (μl) of acetone containing 100mg/ml dansyl chloride (Aldrich Chemical Co., Milwaukee, WI.). After 5 minutes of incubation at room temperature, a small amount of precipitate was seen in the tubes.

A small amount of the material from the synthesis, approximately 0.5μl, was applied to a silica gel ascending thin layer chromatography plate, and the

plate was developed with a solution of methanol:
chloroform: acetic acid having the following ratio:
50:20:1.

5   After the solvent had moved 12 centimeters (cm)
up the plate, the plate was photographed under long-wave
ultraviolet (UV) light on a transilluminator using
Polaroid type 667 film and an orange filter (Tiffen Co.).
The reactions containing the peptide each contained a new
fluorescent product with a mobility slower than the
10   species seen in the control reactions.

         The unique product present in the Kemptide
peptide reaction had a slightly slower mobility than that
seen in the V^6V^5 Kemptide reaction. These unique peptides
are the dansyl derivatives desired from these peptides
15   and were renamed Promega Peptide 1 (SEQ ID NO:1) and
Promega Peptide 2 (SEQ ID NO:2) for the Kemptide and V^6A^5
Kemptide derivative, respectively.

Example 2

Purification of Promega Peptides 1 (SEQ ID NO:1)
and 2 (SEQ ID NO:2)

A column (2.5 x 15cm) of Sephadex G25 fine
5   (Sigma Chemical Co., St. Louis, MO.) was made according
to the recommendations of the manufacturer and
equilibrated using 10% ethanol in 20mM Tris pH 8.0. One
ml of the synthesis solutions for the modified peptides
Promega Peptide 1 (SEQ ID NO:1) or 2 (SEQ ID NO:2) was
10   applied to the column and the column was then eluted
using 10% ethanol, 20mM Tris pH 8.0. Five ml fractions
were collected and 2μls of the fractions were analyzed by
thin layer chromatography and photographed as described
in Example 1.
15   Fractions 8-12 collected from the column were
shown to contain the fluorescent modified peptide
species, but did not contain significant levels of the
unbound, spent derivitization reagent. These fractions
were pooled and lyophilized to dryness to obtain
20   purified, modified peptide pools of Promega Peptides 1
(SEQ ID NO:1) and 2 (SEQ ID NO:2).

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Example 3

Assay of Protein Kinase Using Promega

Peptides 1 (SEQ ID NO:1) and 2 (SEQ ID NO:2)

Radioactive kinase assays were carried out as

described in <u>Walton et al</u>., as modified and described

here. Five μl Promega Peptide 1 (SEQ ID NO:1) were added

to a reaction mixture containing 20mM Tris HCl (pH 7.4),

10 mM MgCl$_2$, 4 micrograms (μg) of cAMP-dependent protein

kinase (both catalytic and regulatory subunits), in the

presence or absence of 2 micromolar (μM) cAMP, in a

volume of 50μl.

The reaction began with the addition of 10μl of

solution containing 0.15mM [γ_{32}P] ATP (about 0.05Ci/mmol)

and 10mM MgCl$_2$. After a two minute incubation, the

reaction was terminated by the addition of 30μl of H$_3$PO$_4$

to a final concentration of 0.5%. To measure

incorporation of radiolabelled phosphate into the peptide

substrate, half the reaction mixture was spotted onto a

Whatmann P81 filter. The filters were washed four times

in a solution containing 0.5% H$_3$PO$_4$. Each wash lasted at

least five minutes. After washing, the radioactivity

present on the filters was measured in a scintillation

counter. Both of the peptides showed increased

phosphorylation in the presence of cAMP, indicating that

they are substrates for cAMP-dependent protein kinase.

The counts per minute (CPMs) resulting from the assays

are given in Table 1 below:.

Table 1

Substrate	CPM (no cAMP)	CPM (+cAMP)	Stimulation
Kemptide	5760	471,840	82
Dansyl Kemptide	3166	393,062	124
Dansyl V^{6}A^5-Kemptide	21044	581,682	28

Example 4

Separation of Phosphorylated Promega
Peptides 1 (SEQ ID NO:1) and 2 (SEQ ID NO:2) from the
Unphosphorylated Peptides by Thin Layer Chromatography

5   Promega Peptides 1 (SEQ ID NO:1) and 2 (SEQ ID
NO:2) were phosphorylated as described in Example 3 using
non-radioactive ATP in a final volume of $60\mu l$. Samples
from these reactions were spotted onto Whatman Silica Gel
60 A thin-layer chromatography plates (Sigma Chemical
10   Co., St. Louis, MO.), and the spots were developed with a
solution containing 55% methanol, 40% chloroform, and 5%
acetic acid. Once the solvent front neared the top of
the plates, the plates were dried and examined under a UV
light.

15   Referring now to Fig. 1, the non-phosphorylated
species show the highest mobility, with an R_f of 0.81;
the phosphorylated peptides showed a lower mobility with
an R_f of 0.12.

          Referring to Fig. 1, which is a photograph of a
20   thin-layer chromatography (TLC) plate, the phosphorylated
and non-phosphorylated Promega Peptides 1 (SEQ ID NO:1)
and 2 (SEQ ID NO:2) have been spotted.

          Lane 1: contains 2λ of non-phosphorylated
Promega Peptide 1 (SEQ ID NO:1).

25   Lane 2: contains 5λ of phosphorylated Promega
Peptide 1 (SEQ ID NO:1).

          Lane 3: contains 2λ of non-phosphorylated and
5λ of phosphorylated Promega Peptide 1 (SEQ ID NO:1).

          Lane 4: contains 2λ of non-phosphorylated
30   Promega Peptide 2 (SEQ ID NO:2).

          Lane 5: contains 5λ of phosphorylated Promega
Peptide 2 (SEQ ID NO:2).

          Lane 6: contains 2λ of non-phosphorylated and
5λ of phosphorylated Promega Peptide 2 (SEQ ID NO:2).

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Example 5

Separation of Phosphorylated Promega

Peptides 1 (SEQ ID NO:1) and 2 (SEQ ID NO:2)

from the Unphosphorylated Peptides by Electrophoresis

5   Promega Peptides 1 (SEQ ID NO:1) and 2 (SEQ ID NO:2) were phosphorylated as described in Example 3 with the following modifications:

          1)   no radioactivity was used;

          2)   incubation time was 1 hour; and

10   3)   the reactions were stopped by quick freezing of the reaction by placing the tube containing the reaction in a dry ice/ethanol bath.

          Glycerol was added to a final concentration of 5%, and 10μl of each of the reactions (approximately 4μl

15   of peptide) were loaded onto a 0.8% agarose submarine gel, equilibrated with 50mM Tris HCl, pH 8.0. The gel was run for one hour at 50 volts (V). Complete separation of the two species was observable fifteen minutes after starting the gel. After completion, the

20   gel was viewed by a fluorometer to illuminate the dansylated peptides.

          Reference is made to Fig. 2, which is a photograph of 0.8% agarose gel used to separate phosphorylated and non-phosphorylated Promega Peptides 1

25   (SEQ ID NO:1) and 2 (SEQ ID NO:2). The anode end of the gel is to the right:

          Lane 3:   contains non-phosphorylated Promega Peptide 1 (SEQ ID NO:1).

          Lane 4:   contains Promega Peptide 1 (SEQ ID

30   NO:1) that has been partially phosphorylated.

          Lane 5:   contains partially phosphorylated Promega Peptide 2 (SEQ ID NO:2).

          Lane 6:   contains non-phosphorylated Promega Peptide 2 (SEQ ID NO:2).

35   The non-phosphorylated peptides 1 (SEQ ID NO:1) and 2 (SEQ ID NO:2), which have a n t change of +1, migrated to the left of the origin. The phosphorylated

peptide, which has a net change of -1, migrated to the right. Bands on the far right side are due to dansyl dye that did not react with the peptide during synthesis.

Example 6
Detection of Small Amounts of Protein Kinase
Using Promega Peptide 1 (SEQ ID NO:1)

Promega Peptide 1 was phosphorylated under non-radioactive conditions as described in Example 5 with varying amounts of cAMP-dependent protein kinase for 15 minutes. The reactions were stopped by heating the reaction samples at 95°C for 10 minutes. Glycerol was added to a final concentration of 1%, and 20μl of each reaction mixture were loaded onto a 0.8% agarose submarine gel. The gel was run for 1 hour at 75V, and dansylated peptide was detected by UV light.

The phosphorylated peptide could be detected in the reaction mixture that had been incubated with 5 nanograms (ng) of cAMP-dependent protein kinase, and intensity of the phosphorylated band increased with the amount of kinase that had been present in the reaction mixture.

Reference is made to Fig. 3, which is a photograph of a 0.8% agarose gel used to determine the detection limit of cAMP-dependent protein kinase (CAPK) using Promega Peptide 1 (SEQ ID NO:1):

Lane 1: contains 25μg Promega Peptide 1 (SEQ ID NO:1) and no cAMP-dependent kinase.

Lane 2: contains 25μg Promega Peptide 1 (SEQ ID NO:1) and 225ng CAPK.

Lane 3: contains 25μg Promega Peptide 1 (SEQ ID NO:1) and 45ng CAPK.

Lane 4: contains 25μg Promega Peptide 1 (SEQ ID NO:1) and 5ng CAPK.

Lane 5: contains 25μg Promega Peptide 1 (SEQ ID NO:1) and 20ng CAPK.

Lane 6: contains 25μg Promega Peptide 1 (SEQ ID NO:1) and 2.5ng CAPK.

Lane 7: contains 25μg Promega Peptide 1 (SEQ ID NO:1) and .25ng CAPK.

The non-phosphorylated Promega Peptide 7 (SEQ ID NO:7) migrated to the left of the origin. The phosphorylated Promega Peptide 7 (SEQ ID NO:7) migrated to the right of the origin. Bands on the far right are
5   due to free dye that did not react with the peptide during synthesis.

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Example 7
Detection of Protein Kinase Activity in
Crude Extracts using Promega Peptide 1 (SEQ ID NO:1)

Crude extracts were prepared from rat brain as
described in Example 14 (_infra._) without applying the
sample to the DEAE column. Ten μl of diluted homogenate
were incubated with protein kinase C reaction buffer
(described in Example 13, _infra._), 10μl Promega Peptide 1
(SEQ ID NO:1), and 2μM cAMP in a final volume of 100μl
for a time ranging between 0 seconds to 10 minutes.

Reactions were stopped by heating to 95°C for
10 minutes. Glycerol was added to 4%, and 10μl of the
solutions were applied to an 8% agarose gel as described
in Example 6 (_supra._). Visualization of phosphorylated
peptide was achieved by viewing the gel under UV light
and could be seen within 10 minutes.

The cAMP-dependent protein kinase was
detectable at all incubation times.

Reference is made to Fig. 4, which is a
photograph of a 0.8% agarose gel showing the separation
of non-phosphorylated Promega Peptide 1 (SEQ ID NO:1)
from Promega Peptide 1 (SEQ ID NO:1) phosphorylated by
cAMP-dependent protein kinase present in homogenated rat
brain as described in Example 7. The positively-charged
anode end of the gel is on the left.

Lane 3 shows Promega Peptide incubated with
homogenate for 15 seconds. Phosphorylated peptide
migrates to the left, non-phosphorylated peptide to the
right. The other lanes are not relevant to the
experiment.

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Example 8
Synthesis of Promega Peptide 3 (SEQ ID NO:3)
(Sulforhodamine 101 Derivative of V^6A^5 Kemptide)

The peptide V^6A^5 Kemptide was dissolved in 200mM

5   sodium borate buffer, pH 9.0, and 200μl of this solution
was placed in a 1.5ml Eppindorf tube. To this tube was
added 500μl of acetonitrile containing 10mg
sulforhodamine 101 acid chloride (Aldrich Chem. Co.,
Milwaukee, WI.). The reaction mixture was allowed to

10 incubate for 10 minutes at room temperature, and 300μl of
water was added to the tube.

An agarose gel (1% w/w agarose in 20mM Tris
hydrochloride, pH 8.0) was prepared by suspending the
agarose in the buffer and heating to boiling in a

15 microwave. The solution was mixed by stirring, and the
molten agarose was poured into a BioRad horizontal gel
apparatus (Biorad Co., Richmond CA., Cat. No. 170-4307)
containing a comb to provide sample slots.

After the solution had solidified by cooling to

20 room temperature, the comb was removed from the gel and a
gel approximately 0.5cm thick was produced. Twenty
microliters of the reaction mixture described above was
added to 5μl of 80% glycerol, and the mixture was added
to one of the loading wells in the gel. The gel was

25 placed in the running apparatus of the manufacturer and
20mM Tris hydrochloride, pH 8.0 was added as a running
buffer according to the instructions of the manufacturer.
Electrophoresis was performed at 200V for 40 minutes.

One colored species, which represents spent

30 sulforhodamine 101 acid chloride, was seen migrating
towards the positive electrode, while a second species
which was composed of the modified peptide, was seen
migrating with a slightly slower mobility towards the
negative electrode. Thus this analysis confirmed that

35 the Sulforhodamine 101 acid chloride derivative of V^6A^5
Kemptide had been synthesized. The peptide was renamed
Promega Peptide 3 (SEQ ID NO:3).

Reference is made to Fig. 5, which is a photograph of the agarose gel used to confirm the synthesis of Promega Peptide 3 (SEQ ID NO:3) photographed under UV light showing the separation of the components

5   present in modification reactions made during synthesis of Promega Peptide 3 (SEQ ID NO:3). The gel was photographed such that the end of the gel oriented towards the negative electrode is located at the top of the photograph and the lanes are numbered from left to

10   right. The samples in the lanes are as follows:

Lanes 1 and 2: no sample;

Lane 3: $20\mu l$ of the synthetic reaction listed in Example 8 describing the synthesis of Promega Peptide 3 (SEQ ID NO:3);

15   Lane 4: an equivalent sample of a mock reaction made as described in Example 8 but lacking the peptide substrate--this reaction allows the mobility of the spent fluorescent reagent to be measured and can be seen as the highly fluorescent species migrating towards

20   the positive electrode;

Lane 5-end: not relevant to this discussion.

The fluorescent species present in Lane 2 which migrates towards the negative electrode is Promega Peptide 3 (SEQ ID NO:3) which has been synthesized in the

25   reaction described in Example 8.

Example 9

Purification of Promega Peptide 3 (SEQ ID NO:3)

The solution of Promega Peptide 3 (SEQ ID NO:3) was applied to a 2.5x15 cm column of Sephadex G25 equilibrated with 50mM ammonium bicarbonate pH 7.8. The column was eluted with 50mM ammonium bicarbonate, and fractions of approximately 8ml were collected. Twenty-five microliters of these fractions was added to 10μl of 80% glycerol. Twenty microliters of these samples were applied to an agarose gel made as described in Example 8 and the gel was run as described in that example.

Those fractions which contained the modified peptide, as demonstrated by containing the colored species which migrated towards the negative electrode, were pooled and lyophilized to produce purified Promega Peptide 3 (SEQ ID NO:3). A solution of this material was made by dissolving the resulting material in 0.5ml distilled water.

Reference is made to Fig. 6, which is a photograph of the agarose gel used to analyze the components present in samples of column fractions collected during purification of Promega Peptide 3 (SEQ ID NO:3) by fractionation of the reaction mixture on Sephadex G25. The gel was photographed under UV light as described in Example 1, and the gel is oriented such that the region of the gel oriented towards the negative electrode is towards the top of the photograph. The lanes are numbered from left to right. The lanes showing no fluorescent species will be ignored and not numbered:

Lane 1:   a sample of fraction 5 (for details on the composition of the samples, see example 8);

Lane 2:   a sample of fraction 6;

Lane 3:   a sample of fraction 7;

Lane 4:   a sample of fraction 8;

Lane 5:   a sample of fraction 9;

Lanes 6-end: not pertinent to this discussion. The samples in lanes 1-5 contained the

fluorescent speci s which migrated towards the negative electrode that was identified in Example 8 as Promega Peptide 3 (SEQ ID NO:3). These fractions were further treated as described in Example 8.

Example 10
Assay of Protein Kinase Using Promega
Peptide 3 (SEQ ID NO:3)

Fifteen microliters of Promega Peptide 3 (SEQ

5   ID NO:3) were incubated with reaction buffer containing 20mM HEPES (pH 7.4), 50mM MgCl$_2$, 15mM ATP, and 2μg cAMP-dependent protein kinase for 2 hours at room temperature. The reaction was stopped by incubating the reaction mixtures for 10 minutes at 95°C. Glycerol was added to a

10   final concentration of 4%, and 10μl of the reaction were loaded on to an 8% agarose gel as described in Example 6.

Electrophoresis of the peptide showed that the phosphorylated species could be separated from the non-phosphorylated species. In addition, the separation

15   could be seen visibly as well as fluorescently. The separation was based on the difference in the charges on the two species as described in Example 5. The separation was visible within ten minutes of the beginning of the electrophoresis.

20   Reference is made to Fig. 7, which is a photograph of an agarose gel showing the separation of phosphorylated and non-phosphorylated Promega Peptide 7 (SEQ ID NO:7). The anode end of the gel is on the right.

Lane 1: non-phosphorylated Promega Peptide 3

25   (SEQ ID NO:3);

Lane 2: Promega Peptide 3 (SEQ ID NO:3) that had been incubated with cAMP-dependent protein kinase for 30 minutes at room temperature (23°C);

Lanes 3-8: not relevant to this example.

30   The positively charged non-phosphorylated peptide migrated to the left of the origin. The phosphorylated peptide migrated to the right.

Example 11
Synthesis of Promega Peptides 4 (SEQ ID NO:4),
5 (SEQ ID NO:5), 6 (SEQ ID NO:6)

The peptide listed [ser25] protein kinase C

5   (Bachem Inc., Torrence, CA., Cat. No. PPHA115) was
dissolved in distilled water to a concentration of
10mg/ml. Two 100μl samples of this solution were placed
in 1.5ml Eppindorf tubes with 600μl water and 100μl 200mM
sodium borate, pH 9.0. This solution was mixed by

10   vortex, and 200μl of 10mg/ml lissamine rhodamine sulfonyl
chloride, (Molecular Probes, Eugene, OR.) in acetonitrile
was added to the reaction. After 15 minutes of
incubation at room temperature, an additional 100μl
borate buffer and 200μl lissamine rhodamine in

15   acetonitrile was added to one of the 1.5ml Eppindorf
tubes. The tubes were allowed to incubate for an
additional 30 minutes at room temperature.

Five and 10μl samples of the 2 reactions
described above were applied to a 0.8% agarose gel made

20   and equilibrated in 50mM Tris-Cl buffer, pH 8.0. The gel
was electrophoresed at 100V for 1 hour, and photographed
on a transilluminator as described above.

The reaction mixture which was given a single
addition of lissamine rhodamine showed a unique band with

25   a high mobility towards the negative electrode which was
assigned the name Promega Peptide 4 (SEQ ID NO:4). The
samples from the reaction given two lissamine additions
did not show this peptide but apparently did contain
unique colored species which moved towards the positive

30   electrode. The nature of these species was not
determined.

Reference is made to Fig. 8, which is a
photograph of the agarose gel used to analyze the
components present in samples of the reaction mixes

35   tested for production of Promega Peptide 4 (SEQ ID NO:4).
The gel was photographed under UV light as described in
Example 1 and the gel is oriented such that the region of

the gel oriented towards the negative electrode is towards the top of the photograph. The lanes are numbered from left to right:

Lane 1: 5µl reaction mix given a single lissamine rhodamine addition (see example 11 for a full description of the samples);

Lane 2: 5µl reaction mix given a second lissamine rhodamine addition;

Lane 3: blank;

Lane 4: 10µl reaction mix given a single reagent addition;

Lane 5: 10µl reaction mix given a second reagent addition.

The fluorescent material seen having a mobility towards the negative electrode greater than that seen for the spent reagent (which migrates towards the positive electrode) in lanes 1 and 4 was identified as Promega Peptide 4 (SEQ ID NO:4).

The peptide H-9685, (Bachem Bioscience Inc., Philadelphia, PA.) was dissolved in water to a concentration of 10mg/ml. Fifty microliters of this material was mixed with 300µl water and 50µl 200mM sodium borate buffer, pH 9.0. One hundred microliters of 10 mg/ml lissamine rhodamine sulfonyl chloride (Molecular Probes, Eugene, OR.), dissolved in acetonitrile, was added to the solution. The reaction was incubated at room temperature for 30 minutes. Four microliters of the material were applied to an agarose gel (0.8% w/v) in 10mM Tris-Cl, pH 8.0. The gel was run as above using 10mM Tris-Cl as a running buffer and photographed on a transilluminator as described above. The gel contained one species which rapidly migrated towards the negative electrode which was named Promega Peptide 5 (SEQ ID NO:5).

Reference is made to Fig. 9, which is a photograph of the agarose gel used to analyze the components present in samples of the reaction mixes

tested for production of Promega Peptide 5 (SEQ ID NO:5).
The gel was photographed under UV light as described in
Example 1 and the gel is oriented such that the region of
the gel oriented towards the negative electrode is
5   towards the top of the photograph. The lanes are
numbered from left to right:
        Lane 1: sample of the reaction mix involved in
the synthesis of Promega Peptide 7 (SEQ ID NO:7)
described in Example 20;
10   Lane 2: sample of the reaction mix involved in
the synthesis of Promega Peptide 5 (SEQ ID NO:5);
        Remaining lanes: not involved.
        The fluorescent species migrating towards the
negative electrode in Lane 1 with a mobility slower than
15   that seen for the spent reagent migrating towards the
positive electrode was identified as Promega Peptide 7
(SEQ ID NO:7); the fluorescent species migrating towards
the negative electrode with a mobility faster than that
seen for the spent reagent migrating towards the positive
20   electrode in Lane 2 was identified as Promega Peptide 5
(SEQ ID NO:5).
        Peptide PPHA117 was dissolved in water to a
concentration of 10mg/ml and duplicate $100\mu l$ samples of
this solution was placed in individual 1.5ml Eppindorf
25   tubes. To these tubes was added $600\mu l$ water and $100\mu l$
sodium borate buffer, pH 9.0. Two hundred microliters of
acetonitrile containing 10mg/ml of lissamine rhodamine
sulfonyl chloride were added to each tube. The tubes
were incubated for 15 minutes at room temperature. At
30   this time, $20\mu l$ 1 molar (M) NaOH were added to one of the
tubes followed by an additional $200\mu l$ lissamine rhodamine
solution in acetonitrile. After an additional 5 minutes,
$5\mu l$ samples of the reaction solution was applied to an
0.8% agarose gel buffered with 50mM Tris-Cl, pH 8.0. The
35   gel was run as described above and photographed on a
transilluminator.

Referring to Fig. 10, the photograph indicates that a rapidly moving fluorescent product was migrating towards the negative electrode. This component was named Promega Peptide 6 (SEQ ID NO:6). Shown in Fig. 10 is a

5 photograph of the agarose gel used to analyze the components present in samples of the reaction mixes tested for production of Promega Peptide 6 (SEQ ID NO:6). The gel was photographed under UV light as described in Example 1 and the gel is oriented such that the region of

10 the gel oriented towards the negative electrode is towards the top of the photograph. The lanes are numbered from left to right:

Lane 1: 5μl of the reaction used for synthesis of Promega Peptide 6 (SEQ ID NO:6) not given sodium

15 hydroxide treatment;

Lane 2: 5μl reaction used for synthesis of Promega Peptide 6 (SEQ ID NO:6) given sodium hydroxide;

Lanes 3-4: not involved in this study;

Lane 5: same as Lane 1 but 10μl of reaction;

Lane 6: same as Lane 2 but 10μl of reaction.

20 The fluorescent species migrating towards the negative electrode in the lanes with a mobility slightly faster than that seen for the mobility of the spent reagent migrating towards the positive electrode was

25 identified as Promega Peptide 6 (SEQ ID NO:6).

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Example 12

Purification of Promega Peptides 4 (SEQ ID NO:4),

5 (SEQ ID NO:5), and 6 (SEQ ID NO:6)

In order to purify the peptides described in

5   Example 11, a 2ml bed volume of SP Trisacryl

(Biotechnics, Inc.) was poured and equilibrated in 20mM

ammonium bicarbonate buffer, pH 7.8. The 2 reaction

mixtures described for synthesis of Promega Peptide 4

(SEQ ID NO:4) were applied to the column. Six 5ml washes

10   of 20mM ammonium bicarbonate buffer were applied to the

column while 5ml fractions were collected. These

fractions eluted the bulk of the spent reagent as

determined by analysis of samples of the fractions by

agarose gel electrophoresis as described above.

15   Ten milliliters of 1M ammonium bicarbonate was

then applied to the column and 1.5ml fractions were

collected. As the 1M buffer migrated through the column,

a second colored species eluted which was shown to be the

derived peptide by agarose gel electrophoresis. The

20   fractions containing this derived peptide were

lyophilized and resuspended in water to produce the stock

pool of Promega Peptide 4 (SEQ ID NO:4).

The stock pools of Promega Peptides 5 (SEQ ID

NO:5) and 6 (SEQ ID NO:6) were made as described above

25   with the exception that Promega Peptide 6 (SEQ ID NO:6)

was eluted from the Trisacryl SP column with 500mM

ammonium bicarbonate, pH 7.8.

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Example 13
Detection of Protein Kinase C Activity
Using Promega Peptides 4 (SEQ ID NO:4), 5 (SEQ ID NO:5),
and 6 (SEQ ID NO:6)

5   Fifteen microliters of Promega Peptide 5 (SEQ
ID NO:5) were incubated in protein kinase C reaction
buffer (20mM HEPES (pH 7.4), 1.3mM $CaCl_2$, 1mM DTT, 10mM
$MgCl_2$, 200μg/mL phosphatidyl serine, 15mM ATP), and 1μg
Protein Kinase C for 2 hours at room temperature in a

10 final volume of 40μl. The reaction was stopped by
heating the reaction solution to 95°C for ten minutes.
Glycerol was added to a final concentration of 4%, and
10μl solution were loaded on an 8% agarose gel as
described in Example 5.

15   The separation of the phosphorylated and non-
phosphorylated species was based on the difference in
charges as described in Example 5, and could be seen
under UV light within 20 minutes.

              Reference is made to Fig. 11, which is a
20 photograph of a 0.8% agarose gel used to separate
phosphorylated and non-phosphorylated Promega Peptides 4
(SEQ ID NO:4), 5 (SEQ ID NO:5), 6 (SEQ ID NO:6). The
peptides were incubated with varying amounts of protein
kinase C as described in Example 13. The positively

25 charged anode end of the gel is toward the top:
              Lanes 1-6: contain Promega Peptide 4 (SEQ ID
NO:4).
              Lanes 7-12: contain Promega Peptide 5 (SEQ ID
NO:5).

30   Lanes 13-18: contain Promega Peptide 6 (SEQ ID
NO:6).
         The following data indicate the number of moles of
protein kinase C present during each incubation.
              Lanes 1, 7, 13: 730×10^{-15} moles PKC (1 μg)

35   Lanes 2, 8, 14: 145×10^{-15} moles PKC
              Lanes 3, 9, 15: 14.5×10^{-15} moles PKC
              Lanes 4, 10, 16: 7.25×10^{-15} moles PKC

Lanes 5, 11, 17:   1.45 x 10^{-15} moles PKC
Lanes 6, 12, 18:   730 x 10^{-18} moles PKC

Example 14

Detection of Protein Kinase C Activity in

Crude Samples Using Promega Peptide 6 (SEQ ID NO:6)

The brains from 100 adult male rats were

5   obtained from Harlan Sprague Dawley (Indianapolis, IN.).

The brains were homogenized with a PolyTron (Model SDT

1810, Tekmar) in 250ml buffer containing 20mM Tris buffer

(pH 7.5), 10mM EGTA, 2mM EDTA, 5mM DTT, 1mM PMSF, 10mg/L

Leupeptin, and 10mg/L aprotinin. The homogenate was

10   centrifuged for 20 minutes at 9000 x g and filtered

through glass wool. The liquid portion was then adjusted

to pH 7.9, and diluted with water until the conductance

was less than 0.8mmho. The diluted solution was then

applied to a 250mL DEAE anion exchange column

15   equilibrated in 20mM Tris (pH 7.5), 2mM EDTA, 2mM EGTA,

and 1mM DTT. After loading, the column was washed with 1

liter (L) of buffer, and protein was eluted in a linear

salt gradient ranging from 0.0M NaCl to 0.4M NaCl.

Column fractions were assayed for protein

20   kinase C activity by two methods: radioactively, which

is known to the art and described by Walton et al., 1987,

and non-radioactively as described below: 10μl Promega

Peptide 6 (SEQ ID NO:6) were incubated with protein

kinase C reaction buffer (described in Example 13) for 30

25   minutes at room temperature in a final volume of 40μl.

The reactions were stopped by heating the reaction

mixtures to 95°C for ten minutes. Glycerol was added to

a final concentration of 4%, and 25μl were applied to an

8% agarose gel, which was run as described in Example 5.

30   The phosphorylated peptide was visualized under

UV light and could be distinguished from non-

phosphorylated peptide within 15 minutes. Comparison of

the results of the non-radioactive assay with the results

of the radioactive assay showed that protein kinase C

35   detected in fractions by 1 method was reliably detected

by the other method.

Reference is made to Fig. 12, which is a
photograph of a 0.8% gel used to detect the presence of
protein kinase C in DEAE column fractions. Partial
purification of the enzyme and reaction conditions are
5   described in Example 14. The positively charged anode
end of the gel is toward the top. The following data
indicates the contents of the lanes of the gel.

Lane	Contents	Lane	Contents
1	Blank	10	39
2	Homogenate	11	42
3	Blank	12	45
4	Blank	13	48
5	Fraction 10	14	51
6	"F" 20	15	55
7	F 30	16	60
8	F 30	17	70
9	F 36		

The non-phosphorylated peptide migrated to the bottom of
the gel. The phosphorylated peptide, which had a net
20   charge of zero, remained at the origin.

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Example 15

Detection of Small Amounts of Protein Kinase C

Using Promega Peptides 4 (SEQ ID NO:4), 5 (SEQ ID NO:5)

and 6 (SEQ ID NO:6)

5   Five microliters of Promega Peptide 5 (SEQ ID
NO:5) and 6 (SEQ ID NO:6) or 2.5μl of Promega Peptide 4
(SEQ ID NO:4) were incubated with a solution containing
18μl protein kinase C reaction buffer (described in
Example 13) and decreasing amounts of protein kinase C,

10   containing between 60ng and 60 picograms (pg), for 30
minutes at room temperature. Reactions were stopped by
heating the reaction mixtures to 95°C for 10 minutes.
Glycerol was added to a final concentration of 4%, and
25μl of the solution were applied to an 8% agarose gel as

15   described in Example 5.

          Referring to Fig. 11, the detection of
phosphorylated peptide was observed under UV light, and
detection limits noted. Six hundred picograms of protein
kinase C could be detected by Promega Peptide 4 (SEQ ID

20   NO:4), 3ng by Promega Protein 5 (SEQ ID NO:5), and 3ng by
Promega Protein 6 (SEQ ID NO:6).

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Example 16

Assay of Pure Proteases Using Promega P ptides

The proteases Modified Trypsin [sequencing grade] (Promega Corp., Madison, WI.) and Endoprotease Lys

5   C [sequencing grade] were dissolved in A buffer (50mM ammonium bicarbonate, pH 7.8). These stocks were diluted with A buffer to produce stocks containing 0.1, 0.01, and 0.0001μg protease per 4μl of liquid. Independent incubations of 4μl of each of the different protease

10   concentrations were performed with Promega Peptides 5 (SEQ ID NO:5), 6 (SEQ ID NO:6), 7 (SEQ ID NO:7) (5μl of the stocks described above) in a 20μl reaction mixture containing: 5μl peptide; 4μl protease; and 11μl A buffer. Control reaction mixtures were made for each of

15   the peptides which had an additional 4μl of A buffer added in place of the protease.

After 90 minutes at 37°C, the samples were loaded into a 0.8% agarose gel made in 20mM Tris, pH 8.0. The samples were electrophoresed at 150V. When the

20   control peptide sample had migrated about 0.5 inch from the loading well, the gels were photographed on a transilluminator as described above. The incubations made with trypsin displayed new peptide species as revealed by their mobility at all protease level and for

25   all peptides as was expected if the protease could digest such peptides when labeled with the detection tag.

Reference is made to Fig. 13, which is a photograph of the agarose gel used to analyze the peptide species produced in the digestion of Promega Peptide 6

30   (SEQ ID NO:6) by modified trypsin and Endoprotease Lys C. The gel was photographed under UV light as described in Example 1 and the gel is oriented such that the end of the gel which was oriented towards the negative electrode is towards the top of the photograph. The lanes are

35   numbered from left to right. Lane 1 identifies a control reaction sample which did not contain any added protease (see Example 16 for a more complete description of the

samples if desired); Lane 2, a sample of the digestion of Promega Peptide 6 (SEQ ID NO:6) by 0.1µg Endoprotease Lys C; Lane 3, a sample of the digestion of Promega Peptide 6 (SEQ ID NO:6) by 0.01µg Endoprotease Lys C; Lane 4, a

5   sample of the digestion of Promega Peptide 6 (SEQ ID NO:6) by 0.001µg Endoprotease Lys C; Lane 5, a sample of the digestion of Promega Peptide 6 (SEQ ID NO:6) by 0.1µg Modified Trypsin; Lane 6, a sample of the digestion of Promega Peptide 6 (SEQ ID NO:6) by 0.01µg Modified

10   Trypsin; and Lane 7, a sample of the digestion of Promega Peptide 6 (SEQ ID NO:6) by 0.001µg Modified Trypsin. Note that fluorescent species with mobilities altered from that seen for Promega Peptide 6 (SEQ ID NO:6) are seen in the lanes containing samples from incubation of

15   the peptide with moderate and high levels of added protease.

These results indicate that this method cannot only be used to assay proteases but a panel of peptides can be used to indicate the specificity of an unknown

20   protease.

Reference is made to Fig. 14, which is a photograph of the agarose gel used to analyze the peptide species produced in the digestion of Promega Peptides 5 (SEQ ID NO:5) and 7 (SEQ ID NO:7) by Modified Trypsin and

25   Endoprotease Lys C. The gel was photographed under UV light as described in Example 1 and the gel is oriented such that the end of the gel which was oriented towards the negative electrode is towards the bottom of the photograph. The lanes are numbered from left to right.

30   The first seven lanes containing fluorescent species contain Promega Peptide 5 (SEQ ID NO:5) samples and the second seven lanes containing fluorescent species contain the Promega Peptide 7 (SEQ ID NO:7) samples. The samples in the lanes comprising the first set of seven lanes

35   containing fluorescent species are: Lane 1, control incubation without protease; Lanes 2, 3 and 4, incubations including 0.1, 0.01, and 0.001µg Endoprotease

Lys C, and 0.001μg Modified Trypsin, respectively; Lanes
5, 6 and 7, incubations including 0.1, 0.01, and 0.001μg
Modified Trypsin, respectively. The samples in the
second set of seven lanes containing fluorescent species
5   are in the same relative order as described for the first
set of seven lanes described above.

         The samples incubated with Endoprotease Lys C
did not display as large an amount of product peptide
formed as did the trypsin incubated samples and the
10   Promega Peptide 7 (SEQ ID NO:7) samples did not show any
digestion by Endoprotease Lys C.. The lack of digestion
of the Promega Peptide 7 (SEQ ID NO:7) by Endoprotease
Lys C was expected since no predicted digestion site for
the protease was in the peptide.

Example 17.

Assays of Proteases in Crude Media Samples

Samples of culture supernate from stationary phase cultures of <u>Thermus aquaticus</u> (<u>T. aquaticus</u>) and <u>Brevibacterium albidum</u> (<u>B. albidum</u>) were made by centrifugation of a stationary phase culture at 12,000xg for 2 minutes and transfer of the supernate to fresh tubes. Diluted stocks of Promega Peptides 3 (SEQ ID NO:3), 5 (SEQ ID NO:5) and 6 (SEQ ID NO:6) were made by dilution of the original stocks of these peptides with an equal volume of 50mM ammonium bicarbonate, pH 7.8. In three independent tubes per peptide, $10\mu L$ of these diluted stocks were incubated with either $10\mu L$ water, $10\mu L$ Thermus supernate, or $10\mu L$ Brevibacterium supernate. The incubations were performed for 90 minutes at 37°C. The samples were then loaded in a 0.9% agarose gel made with 20mM Tris-Cl pH 8.0. The gel was then electrophoresed at 120V for 30 minutes, and was photographed under UV light on a transilluminator as described above.

Reference is made to Fig. 15, which is a photograph of the agarose gel used to analyze the peptide species produced in the digestion of Promega Peptides 3 (SEQ ID NO:3), 5 (SEQ ID NO:5), and 6 (SEQ ID NO:6) by supernates of cultures of <u>T. aquaticus</u> and <u>B. albidum</u>. The gel was photographed under UV light as described in Example 1 and the gel is oriented such that the end of the gel which was oriented towards the negative electrode is towards the top of the photograph. The lanes are numbered from left to right:

Lanes 1-4,8 (top tier): blank;

Lane 5 (top tier): sample from the incubation of Promega Peptide 5 (SEQ ID NO:5) with a <u>B. Albidum</u> supernate;

Lane 6 (top tier): sample from the incubation of Promega Peptide 5 (SEQ ID NO:5) with a <u>T. aquaticus</u> supernate;

Lane 7:   control incubation of Promega Peptide 5 (SEQ ID NO:5) without supernate.

The peptide species were produced by incubation of the peptide with the supernates indicating that proteolysis of the peptide had taken place.

Lane 1-2 (lower tier):   blank;

Lane 3 (lower tier):   sample from the incubation of Promega Peptide 3 (SEQ ID NO:3) with supernate from B. albidum supernate;

Lane 4 (lower tier):   sample of Promega Peptide 3 (SEQ ID NO:3) with supernate from T. aquaticus supernate;

Lane 5 (lower tier):   sample from a control incubation of Promega Peptide 3 (SEQ ID NO:3) without supernate;

Lane 6 (lower tier):   sample of the incubation of Promega Peptide 6 (SEQ ID NO:6) with B. albidum supernate;

Lane 7 (lower tier):   sample from the incubation of Promega Peptide 6 (SEQ ID NO:6) with T. aquaticus supernate;

Lane 8 (lower tier):   sample from a control incubation of Promega Peptide 6 (SEQ ID NO:6) without supernate.

The new fluorescent species were observed in lanes 4, 6, and 7, indicating that proteolytic cleavage of the peptide had occurred. Subjecting the gel to prolonged electrophoresis also revealed that the sample in lane 3 did indeed contain a novel peptide species made in small amount which had a slightly faster mobility than the starting peptide.

The gel revealed that new peptide species were generated from the peptides and that the pattern of peptides formed and their amounts were dependent upon the extract used. In addition, the control incubations indicated that the peptides were stable to incubation under these conditions in the absence of protease. Thus

these results indicate that such incubations can be used to detect proteases in crude samples and can be used to begin to characterize the types of proteases present in such samples.

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Example 18

Quantitation of cAMP-dependent Protein Kinase
Activity Using Microtiter Plate Reader

Eight microliters of Promega Peptide 7 (SEQ ID

5   NO:7) was phosphorylated by the catalytic subunit of the
cAMP-dependent protein kinase. The amount of kinase used
in the assay varied from 4.5µl to 0.9ng, diluted from a
stock solution of 2.25mg/ml. The reactions took place in
a volume of 24µl for 30 minutes at room temperature, and

10   were stopped by heating the solutions at 95°C for five
minutes.

After heating, glycerol was added to the
solutions to a final concentration of 4%. The solutions
were then loaded on a 0.8% agarose gel, which was run at

15   100V until the phosphorylated and non-phosphorylated
bands had separated and migrated from the wells.

The agarose containing the phosphorylated
peptide was excised from the gel with a razor blade and
placed into Eppendorph tubes. The agarose was melted,

20   and 145µl agarose solution were diluted in urea and
acetic acid to a final concentration of 2M and 1M,
respectively, in a final volume of 250µl.

The solutions were placed in the wells of a
microtiter plate and allowed to cool. After the agarose

25   re-solidified, the absorbance of the wells at 570
nanometers (nm) was read. The results are as listed
below in Table 3:

Table 3

#	Dilution	Kinase (mg)	A_{570}	A_{570} - Blank
30 1-2	0	0	0.097+/-0.010	0.000
3-4	1:10,000	0.0009	0.106+/-0.013	0.009
5-6	1:1,000	0.009	0.107+/-0.003	0.010
7-8	1:100	0.09	0.119+/-0.002	0.022
9-10	1:10	0.9	0.204+/-0.003	0.107
35 11-12	1:2	4.5	0.203+/-0.005	0.106

In the range of 0.009 to 0.9µg kinase added,
the correlation coefficient was 0.999, indicating a
linear relationship between kinase concentration and
absorbance. Under the conditions in which the assay was

run, more than 0.9μg kinase saturated the system, while less than 0.009μg could not be detected.

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Example 19
Quantitation of cAMP-dependent Protein Kinase
Activity Using a Spectrophotometer

Three microliters of Promega Peptide 7 (SEQ ID
5   NO:7) were phosphorylated by varying amounts of cAMP-
dependent protein kinase. The reactions took place in a
volume of 20μl in a solution containing 3mM ATP, 20mM
HEPES buffer (pH 7.4), and between 50ng and 1μg catalytic
subunit of cAMP-dependent protein kinase. The mixture
10 was incubated for 30 minutes at room temperature, and the
reactions were stopped by heating the mixture at 95°C for
five minutes.

Glycerol was added to a final concentration of
4%, and the mixtures were applied to a 0.8% low melting-
15 point agarose gel (FMC), which was run at 100V for 10
minutes. At this time, the phosphorylated and non-
phosphorylated species had separated and could be
detected by ultraviolet light. The phosphorylated
peptide was then excised from the gel with a razor blade,
20 and the agarose was melted by heating at 95°C for 2
minutes. Two hundred microliters of the melted agarose
were then diluted to 400μl in a solution containing 2M
urea and 1M acetic acid. The absorbance of the solution
at 570nm was then determined in a Milton-Roy Spectronic
25 1201 spectrophotometer.

Reference is made to Table 4 as follows for the
results:

Table 4

	#	Kinase Dilution	Kinase (mg)	A_{570}	A_{570}-Blank
30	1-2	0 (blank)	0	0.015	0.000
	3-4	1:200	0.05	0.024	0.009
	5-6	1:100	0.10	0.036	0.021
	7-8	1:50	0.20	0.044	0.029
	9-10	1:20	0.50	0.145	0.130
35	11-12	1:10	1.0	0.178	0.163

Example 20.

Synthesis and Purification of Promega

Peptide 7 (SEQ ID NO:7)

A 50μl solution of Kemptide (Sigma Co.) in
200mM sodium borate buffer, pH 9.0, was added to 350μl
distilled water. One hundred microliters of acetonitrile
containing 10mg/ml lissamine rhodamine sulfonyl chloride
(Molecular Probes) was added. The solution was incubated
for 5 minutes, and then agarose gel electrophoresis and
visualization of the fluorescent components was performed
as described in Example 8.

Referring the Fig. 16, lane 1, one fluorescent
species was found which was migrating towards the
positive electrode. This species was identified as
Promega Peptide 7 (SEQ ID NO:7). The other lanes were
not relevant to this example.

The remaining amount of the synthetic material
was applied to a 1ml bed volume of SP Trisacryl (LKB
Corp.) equilibrated with 20mM ammonium bicarbonate, pH
7.8. The column was washed with 20mM ammonium
bicarbonate, pH 7.8, until the highly colored material
which corresponds to the spent derivatizing reagent
eluted from the column. One milliliter of 100mM sodium
chloride in 20mM ammonium bicarbonate, pH 7.8, was then
applied to the column followed by several milliliters of
200mM sodium chloride in 20mM ammonium bicarbonate, pH
7.8. A colored species eluted from the 200mM sodium
chloride washes which was identified as Promega Peptide 7
(SEQ ID NO:7) by agarose gel electrophoresis as described
above. This material was lyophilized to dryness and
resuspended in 0.5ml of distilled water to form the
solution of Promega Peptide 7 (SEQ ID NO:7).

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Example 21

Assay of HIV Protease Using Modified Peptide Substrates

　　　In order to assay the HIV protease using the
method employing modified peptide substrates, one of the
5　　two following peptides can be dissolved in water to a
concentration of 10mg/ml in water:

　　　　　PEPTIDE A　R-S-L-N-Y-P-Q-S-U-W

　　　　　PEPTIDE B　A-T-L-N-F-P-I-S-P-W

　　　These solutions can be used in synthetic
10　　reactions having the following compositions:

　　　　　10% (v/v) dissolved peptide

　　　　　10% (v/v) 200mM sodium borate, pH 9.0

　　　　　60% (v/v) distilled water

　　　　　20% (v/v) acetonitrile containing 10mg/ml

15　　　　　　　　lissamine rhodamine sulfonyl chloride

　　　　　　　　(Molecular Probes, Inc.)

　　　The reaction solutions can be incubated at room
temperature for 5 minutes following mixing. At this
time, the solutions will contain a mixture of highly
20　　colored and fluorescent species. These species can be
separated and individually visualized by agarose gel
electrophoresis as described in many previous examples,
such as example 11. The major species will be spent
lissamine rhodamine reagent, the other colored species
25　　will be the modified peptide desired containing a dye
molecule at the amino terminus of the peptide.

　　　Confirmation of the identity of the spent
reagent can be done by fractionation of a reaction
mixture made as described above, but having the peptide
30　　solution added replaced by distilled water. If this is
done, the novel colored species observed by
electrophoretic fractionation of the sample will be the
desired peptide product.

　　　The product from the reaction with peptide A
35　　above will generate Promega Peptide 9 (SEQ ID NO:9),
which will migrate towards the negative electrode in the
gel system described above and which can be purified as

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described from Promega Peptide 7 (SEQ ID NO:7) presented above.

The product from the reaction with peptide B above will generate Promega Peptide 10 (SEQ ID NO:10), which will migrate towards the positive electrode, but at a slower mobility than that observed for the spent reagent. This modified peptide can be purified as described for Promega Peptide 8 (SEQ ID NO:8), which is presented below.

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Example 22
Synthesis and Purification of a Tyrosine Kinase Modified
Peptide Substrate, Promega Peptide 8 (SEQ ID NO:8)

The following peptide, a known substrate for
the protein tyrosine kinase known as the EGF receptor,
was dissolved at a concentration of 10mg/ml in water:

Asp-Arg-Val-Tyr-Ile-His-Pro-Phe

This peptide was reacted with lissamine
rhodamine sulfonyl chloride as described in the previous
example. After 20 minutes at room temperature, 5μl
reaction mixture was electrophoresed on a 0.9% agarose
gel poured using 20mM Tris-Cl pH 8.0. The gel was run at
150V for 30 minutes and was observed and photographed as
described in previous examples.

Reference is made to Fig. 17 for a drawing
depicting a gel run for confirmation of the synthesis of
Promega Peptide 8 (SEQ ID NO:8), and visualized under UV
light. The lanes are numbered from left to right and the
gel was run such that the positive electrode was oriented
toward the upper segment of the gel, i.e., the upper
region of Fig. 17. The samples loaded in the gel were as
follows:

Lane 1: control reaction made as described in
Example 22 but lacking peptide;

Lanes 2-5: 5μl samples of the reaction mix
made as described in Example 22 which included the
peptide.

The new fluorescent species, which was observed
in the lanes containing the peptide, had a slower
mobility than that of the spent reagent which migrated
towards the positive electrode.

Referring to Fig. 17, the photograph revealed
that the reactions contained in a fluorescent species
that migrated towards the positive electrode which was
not present in control reactions made lacking peptide as
described in the previous example. This new species had
a mobility slower than was seen for the spent reagent

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which also migrated towards this electrode. This peptide was named Promega Peptide 8 (SEQ ID NO:8).

A 4ml DEAE sepharose column was poured and equilibrated with 20mM ammonium bicarbonate, pH 7.8. A

5   0.5ml sample of the synthetic mixture was applied to the column, and 5ml of 20mM ammonium bicarbonate was applied to the column. Five milliliter fractions were collected throughout the elution and 5ml samples of 30, 40, 50, 75, 100, 150, and 200mM ammonium bicarbonate, pH 7.8, were

10   applied to the column. After this point, the column was eluted with additional 200mM ammonium bicarbonate, pH 7.8, and additional 5ml fractions were collected.

As 200mM ammonium bicarbonate was applied, two colored species were seen to migrate through the column.

15   The first colored material which eluted was confirmed to be Promega Peptide 8 (SEQ ID NO:8) by agarose gel electrophoresis run as described above. The fractions containing this material were lyophilized and resuspended in 0.5ml of distilled water to form the pool of Promega

20   Peptide 8 (SEQ ID NO:8). This material can now be used to assay the activity of the EGF Receptor by adding the peptide to a solution of active kinase under conditions where the kinase activity of the enzyme is functional, as is known in the art, and by fractionating the resulting

25   mixture of substrate modified peptide (Promega Peptide 8 (SEQ ID NO:8)) from the product modified peptide by agarose gel electrophoresis as described above, or by use of other separation methods well known in the art and listed above.

30   If agarose gel electrophoresis is performed, the product modified peptide will migrate towards the positive electrode with a higher mobility than is observed for the substrate modified peptide.

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Example 23

Detection of Alkaline Phosphatase

Using Non-Radioactive Assay

Promega Peptide 6 (SEQ ID NO:6) was

5   phosphorylated to provide a substrate for alkaline

phosphatase in the following manner: 40μl Promega

Peptide 6 (SEQ ID NO:6) were incubated in a solution

containing 20mM HEPES (pH 7.4), 1.67mM CaCl$_2$, 1mM

dithiothreotol, 10mM MgCl$_2$, 100μg/mL phosphatidylserine,

10   2mM ATP, and 50ng protein kinase C in a final volume of

100μl at 37°C for 1.5 hours. The reaction was stopped by

incubating the mixture at 95°C for 10 minutes.

After phosphorylation, 0.1M Tris (pH 9.5) was added

to a final pH of 9.2. Fifteen microliters of this

15   solution were mixed with between 0.08 to 200 pmoles of

alkaline phosphatase in a final volume of 20μl and

incubated at 37°C for 45 minutes. The reaction was

stopped by freezing the mixtures in a dry ice/ethanol

bath. Glycerol was added to a final concentration of 4%,

20   and the samples were run on a 0.8% agarose gel at 75V for

20 minutes. Dephosphorylation of the phosphorylated

peptide could be seen with as little as 60ng added in the

dephosphorylation reaction.

Reference is made to Fig. 18, which is a

25   photograph of a 0.8% agarose gel depicting the separation

of phosphorylated Promega Peptide 1 (SEQ ID NO:1) and

Promega Peptide 1 (SEQ ID NO:1) dephosphorylated by

alkaline phosphatase as described in this example. The

positively charged anode side of the gel is on the right:

30   Lane 1: 3μl phosphorylated Promega Peptide 1

(SEQ ID NO:1);

Lane 2: 3μl phosphorylated Promega Peptide 1

(SEQ ID NO:1) that had not been incubated with Alkaline

Phosphatase

35   Lane 3: 3μl phosphorylated Promega Peptide 1

(SEQ ID NO:1) that had not been incubated with Alkaline

Phosphatase 30":

Lane 4:   3μl phosphorylated Promega Peptide 1 (SEQ ID NO:1) that had not been incubated with Alkaline Phosphatase 5':

Lane 5:   3μl phosphorylated Promega Peptide 1 (SEQ ID NO:1) that had not been incubated with Alkaline Phosphatase 30'.

Phosphorylated Promega Peptide migrated to the right of the origin; non-phosphorylated peptide migrated to the left. Bands present on the far right are due to free dansyl.

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Example 24
Synthesis and Purification of Promega
Peptide 11 (SEQ ID NO:11)

A solution of Promega Peptide 6 (SEQ ID NO:6)
5   was diluted with ammonium bicarbonate, pH 7.8, to produce
a solution containing 8mg of peptide in 30ml of 100mM
ammonium bicarbonate, pH 7.8. To this solution was added
60μg Endoprotease Lys C (Promega Corp., Madison, WI).
The solution was incubated at 37°C for 2 hours.

10   After this incubation, a second 60μg
Endoprotease Lys C was added and the solution was allowed
to incubate for an additional 2.5 hours. An agarose gel
(0.8% agarose in 10mM Tris pH 8.0) was used to analyze
the digest materials.

15   The gel was electrophoresed as described in
Example 16, and the results seen were very similar to the
results seen for digestion of Promega Peptide 6 (SEQ ID
NO:6) by the protease described in this example where
0.1μg of protease was used to digest the peptide (see

20   lane 2 of Fig. 13). The peptide species with a mobility
approximately 50% of that seen for Promega Peptide 6 (SEQ
ID NO:6) is named Promega Peptide 11 (SEQ ID NO:11).
Also seen was some material which did not migrate out of
the loading wells, this material was not given a Promega

25   Peptide number but is believed to have the sequence Pro-
Leu-Ser-Arg and have the Lissamine Rhodamine chemical
identification tag present on its amino terminus.

The reaction mixture was diluted to 100ml with
distilled water and applied to a 3ml column of SP

30   Trisacryl M. The material, which was bound to the
column, was washed with 5ml of 20mM ammonium bicarbonate,
pH 7.8. At this point, 3ml fractions were first
collected and 100mM ammonium bicarbonate was applied to
the column. A colored species began to elute from the

35   column. The colored material which eluted from the
column was confirmed to contain Promega Peptide 11 (SEQ
ID NO:11) by its mobility on agarose gel electrophoresis

as described above. However, some amount of the material which did not migrate from the wells during electrophoresis was also present in the fractions. This material was pooled and lyophilized. The lyophilized

5   fractions were then redissolved in distilled water to produce the pool of Promega Peptide 11 (SEQ ID NO:11).

Reference is made to Fig. 19, which is a photograph of the agarose gel used to analyze fractions 1-3 of the SP Trisacryl column. The gel was photographed

10   under UV light as described in Example 1. The gel is oriented such that the end of the gel which was oriented towards the negative electrode is towards the bottom of the photograph. The lanes are numbered from left to right as shown:

15   Lane 1: sample of undigested Promega Peptide 6 (SEQ ID NO:6) as a mobility control;

Lane 2: sample of the 5ml, 20mM ammonium bicarbonate wash (20μl);

Lanes 3-5: 10μl sample of fractions 1-3

20   respectively; ·

The species migrating with a mobility approximately one-half that seen for Promega Peptide 6 (SEQ ID NO:6) is Promega Peptide 11 (SEQ ID NO:11) and is the major species in lanes 2-5.

Example 25

Detection of Protein Kinase C Activity Using

Promega Peptide 11 (SEQ ID NO:11)

One hundred microliters of Promega Peptide 11

5   (SEQ ID NO:11) were incubated for 2 hours with 20mM HEPES

buffer (pH 7.4), 1.67mM $CaCl_2$, 200μg/ml phosphatidyl

serine 0.45mM ATP, 10mM $MgCl_2$ and 2.5μg protein kinase C.

Reactions were stopped by heating the mixtures to 95°C

for 5 minutes.   A 20μl solution was brought to 4%

10 glycerol and loaded on a 0.8% agarose gel.   The gel was

run at 100V for 15 minutes.   Phosphorylated Promega

Peptide 11 (SEQ ID NO:11) migrated toward the anode,

which non-phosphorylated peptide migrated toward the

cathode.

15   Reference is made to Fig. 20, which is a

photograph of a 0.8% agarose gel used to separate

phosphorylated Promega Peptide 11 (SEQ ID NO:11) from the

non-phosphorylated species.   The gel was run at 100V for

15 minutes.   The positively charged anode is on the

20 right:

Lane 1:   5μl non-phosphorylated Promega Peptide

11 (SEQ ID NO:11);

Lane 2:   20μl phosphorylated Promega Peptide 11

(SEQ ID NO:11) (as described in Example 24);

25   Lane 3:   blank;

Lane 4:   5μl phosphorylated Promega Peptide 7

(SEQ ID NO:7).

It is understood that the invention is not

confined to the particular construction and arrangements

30 herein illustrated and described, but embraces such

modified forms thereof and come within the scope of the

claims following the bibliography.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

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                       WHITE, DOUGLAS H.

       (ii) TITLE OF INVENTION: NON-RADIOACTIVE KINASE,
                                PHOSPHATASE AND PROTEASE ASSAY

      (iii) NUMBER OF SEQUENCES: 10

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            (A) MEDIUM TYPE: Floppy disk
            (B) COMPUTER: IBM PC compatible
            (C) OPERATING SYSTEM: PC-DOS/MS-DOS
            (D) SOFTWARE: PatentIn Release #1.0, Version
                          #1.25

       (vi) CURRENT APPLICATION DATA:
            (A) APPLICATION NUMBER:
            (B) FILING DATE:
            (C) CLASSIFICATION:

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(2) INFORMATION FOR SEQ ID NO:1:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 7 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: peptide

       (ix) FEATURE:
            (A) NAME/KEY: Binding-site
            (B) LOCATION: 1

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            (D) OTHER INFORMATION: /label= LABEL
                    /note= "LOCATION OF DANSYL DETECTION
                            TAG"
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        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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    (2) INFORMATION FOR SEQ ID NO:2:

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            (B) TYPE: amino acid
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: peptide

        (ix) FEATURE:
            (A) NAME/KEY: Binding-site
            (B) LOCATION: 1
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    (2) INFORMATION FOR SEQ ID NO:3:

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            (B) TYPE: amino acid
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: peptide

        (ix) FEATURE:
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            (B) LOCATION: 1
            (D) OTHER INFORMATION: /label= LABEL
                    /note= "LOCATION OF SULFORHODAMINE 101
                            DETECTION TAG"

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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    Leu Arg Arg Ala Ser Val Ala
    1               5
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(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 13 amino acids
        (B) TYPE: amino acid
5   (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (ix) FEATURE:
        (A) NAME/KEY: Binding-site
        (B) LOCATION: 1
10   (D) OTHER INFORMATION: /label= LABEL
                /note= "LOCATION OF LISSAMINE RHODAMINE
                DETECTION TAG"

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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15   1   5   10

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 11 amino acids
        (B) TYPE: amino acid
20   (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (ix) FEATURE:
        (A) NAME/KEY: Binding-site
        (B) LOCATION: 1
25   (D) OTHER INFORMATION: /label= LABEL
                /note= "LOCATION OF LISSAMINE RHODAMINE
                DETECTION TAG"

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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30   1   5   10

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 12 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

35   (ii) MOLECULE TYPE: peptide

    (ix) FEATURE:
        (A) NAME/KEY: Binding-site
        (B) LOCATION: 1
        (D) OTHER INFORMATION: /label= LABEL

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                    /note= "LOCATION OF LISSAMINE RHODAMINE
                                DETECTION TAG"

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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        1   5   10

    (2) INFORMATION FOR SEQ ID NO:7:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 7 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: peptide

        (ix) FEATURE:
            (A) NAME/KEY: Binding-site
            (B) LOCATION: 1
            (D) OTHER INFORMATION: /label= LABEL
                    /note= "LOCATION OF LISSAMINE RHODAMINE
                                DETECTION TAG"

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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        1   5

    (2) INFORMATION FOR SEQ ID NO:8:

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            (A) LENGTH: 8 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: peptide

        (ix) FEATURE:
            (A) NAME/KEY: Binding-site
            (B) LOCATION: 1
            (D) OTHER INFORMATION: /label= LABEL
                    /note= "LOCATION OF LISSAMINE RHODAMINE
                                DETECTION TAG"

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

        Asp Arg Val Tyr Ile His Pro Phe
        1   5

    (2) INFORMATION FOR SEQ ID NO:9:

        (i) SEQUENCE CHARACTERISTICS:
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            (B) TYPE: amino acid
            (D) TOPOLOGY: linear

         (ii) MOLECULE TYPE: peptide

         (ix) FEATURE:
               (A) NAME/KEY: Binding-site
               (B) LOCATION: 1
               (D) OTHER INFORMATION: /label= LABEL
                      /note= "LOCATION OF LISSAMINE RHODAMINE
                         DETECTION TAG"

         (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

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         1   5

    (2) INFORMATION FOR SEQ ID NO:10:

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               (A) LENGTH: 10 amino acids
               (B) TYPE: amino acid
               (D) TOPOLOGY: linear

         (ii) MOLECULE TYPE: peptide

         (ix) FEATURE:
               (A) NAME/KEY: Binding-site
               (B) LOCATION: 1
               (D) OTHER INFORMATION: /label= LABEL
                      /note= "LOCATION OF LISSAMINE RHODAMINE
DETECTION TAG"

         (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

         Ala Thr Leu Asn Phe Pro Ile Ser Pro Trp
         1   5   10

    (2) INFORMATION FOR SEQ ID NO:11:

         (i) SEQUENCE CHARACTERISTICS:
               (A) LENGTH: 11 amino acids
               (B) TYPE: amino acid
               (D) TOPOLOGY: linear

         (ii) MOLECULE TYPE: peptide

         (ix) FEATURE:
               (A) NAME/KEY: Binding-site
               (B) LOCATION: 1
               (D) OTHER INFORMATION: /label= LABEL
                      /note= "LOCATION OF LISSAMINE RHODAMINE
                         DETECTION TAG"

         (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

         Pro Leu Ser Arg Thr Leu Ser Val Ala Ala Lys
         1   5   10

CLAIMS

What is claimed is:

1. A non-radioactive method of quantitating the presence or activity of an enzyme on a peptide substrate, comprising:

   a.   adding substrate modified peptide to a

5 solution containing the enzyme;

   b.   incubating the enzyme with the substrate modified peptide under conditions where the enzyme is active for a time sufficient to form a product modified peptide;

10   c.   separating the product modified peptide from the substrate modified peptide; and

   d.   measuring the quantity of product modified peptide.

   2. The method of claim 1 wherein the substrate modified peptide is a pure substrate.

   3. The method of claim 1 wherein the substrate modified peptide is a crude substrate.

   4. The method of claim 1 wherein the substrate modified peptide includes a detector segment selected from the group consisting of dansyl, sulforhodamine, lissamine rhodamine (rhodamine B) and fluorescein.

   5. The method of claim 1 wherein the substrate modified peptide is selected from the group consisting of the following peptides: Promega Peptide 1 (SEQ ID NO:1), Promega Peptide 2 (SEQ ID NO:2), Promega Peptide 3 (SEQ

5 ID NO:3), Promega Peptide 4 (SEQ ID NO:4), Promega Peptide 5 (SEQ ID NO:5), Promega Peptide 6 (SEQ ID NO:6), Promega Peptide 7 (SEQ ID NO:7), Promega Peptide 8 (SEQ ID NO:8), Promega Peptide 9 (SEQ ID NO:9), Promega Peptide 10 (SEQ ID NO:10), Promega Peptide 11 (SEQ ID

10 NO:11) and analogs thereof.

   6. The method of claim 1 wherein the enzyme is selected from the group consisting of kinases, phosphatases and proteases.

7.   The method of claim 1 wherein the enzyme is a
kinase.

8.   The method of claim 7 wherein the substrate
modified peptide is selected from the group consisting of
Promega Peptide 1 (SEQ ID NO:1), Promega Peptide 2 (SEQ
ID NO:2), Promega Peptide 3 (SEQ ID NO:3), Promega
Peptide 4 (SEQ ID NO:4), Promega Peptide 5 (SEQ ID NO:5),
5   Promega Peptide 6 (SEQ ID NO:6), Promega Peptide 7 (SEQ
ID NO:7), Promega Peptide 8 (SEQ ID NO:8), Promega
Peptide 11 (SEQ ID NO:11) and analogs thereof.

9.   The method of claim 7 wherein the kinase is
protein kinase C.

10.   The method of claim 9 wherein the substrate
modified peptide is selected from the group consisting of
Promega Peptide 4 (SEQ ID NO:4), Promega Peptide 5 (SEQ
ID NO:5), Promega Peptide 6 (SEQ ID NO:6), Promega
5   Peptide 11 (SEQ ID NO:11) and analogs thereof.

11.   The method of claim 7 wherein the enzyme is
cAMP-dependent protein kinase.

12.   The method of claim 11 wherein the substrate
modified peptide is Promega Peptide 1 (SEQ ID NO:1),
Promega Peptide 2 (SEQ ID NO:2), Promega Peptide 3 (SEQ
ID NO:3), Promega Peptide 7 (SEQ ID NO:7) and analogs
5   thereof.

13.   The method of claim 7 wherein the enzyme is
tyrosine kinase.

14.   The method of claim 13 wherein the substrate
modified peptide is Promega Peptide 8 (SEQ ID NO:8) and
analogs thereof.

15.   The method of claim 1 wherein the enzyme is a
phosphatase.

16.   The method of claim 15 wherein the substrate
modified peptide is selected from the group consisting of
Promega Peptide 1 (SEQ ID NO:1), Promega Peptide 2 (SEQ
ID NO:2), Promega Peptide 3 (SEQ ID NO:3), Promega
5   Peptide 4 (SEQ ID NO:4), Promega Peptide 5 (SEQ ID NO:5),

Prom ga Peptide 6 (SEQ ID NO:6), Promega Peptide 7 (SEQ ID NO:7), Promega Peptide 8 (SEQ ID NO:8), Promega Peptide 9 (SEQ ID NO:9), Promega Peptide 10 (SEQ ID NO:10), Promega Peptide 11 (SEQ ID NO:11) and analogs thereof, wherein the substrate modified peptide is derived from the products of the reaction of a protein kinase with the substrate modified peptide.

17.   The method of claim 15 wherein the enzyme is alkaline phosphatase.

18.   The method of claim 1 wherein the enzyme is a protease.

19.   The method of claim 18 wherein the substrate modified peptide is selected from the group consisting of Promega Peptide 1 (SEQ ID NO:1), Promega Peptide 2 (SEQ ID NO:2), Promega Peptide 3 (SEQ ID NO:3), Promega Peptide 4 (SEQ ID NO:4), Promega Peptide 5 (SEQ ID NO:5), Promega Peptide 6 (SEQ ID NO:6), Promega Peptide 7 (SEQ ID NO:7), Promega Peptide 8 (SEQ ID NO:8), Promega Peptide 9 (SEQ ID NO:9), Promega Peptide 10 (SEQ ID NO:10), Promega Peptide 11 (SEQ ID NO:11) and analogs thereof.

20.   The method of claim 18 wherein the enzyme is HIV protease.

21.   The method of claim 20 wherein the substrate modified peptide is selected from the group consisting of Promega Peptide 9, (SEQ ID NO:9) and Promega Peptide 10 (SEQ ID NO:10) and analogs thereof.

22.   The method of claim 1 wherein the product modified peptide is measured by fluorescence.

23.   The method of claim 1 wherein the product modified peptide is measured by chemiluminescence.

24.   A bioreagent for quantitating the presence or activity of an enzyme, which bioreagent is selected from the group consisting of Promega Peptide 1 (SEQ ID NO:1), Promega Peptide 2 (SEQ ID NO:2), Promega Peptide 3 (SEQ ID NO:3), Promega Peptide 4 (SEQ ID NO:4), Promega Peptide 5 (SEQ ID NO:5), Promega Peptide 6 (SEQ ID NO:6),

Promega Peptide 7 (SEQ ID NO:7), Promega Peptide 8 (SEQ
ID NO:8), Promega Peptid 9 (SEQ ID NO:9), Promega
Peptide 10 (SEQ ID NO:10), Promega Peptide 11 (SEQ ID
NO:11) and analogs thereof.

     25.   A bioreagent for quantitating the presence of
c-AMP dependent kinase, which bioreagent is selected from
the group consisting of Promega Peptide 1 (SEQ ID NO:1),
Promega Peptide 2 (SEQ ID NO:2), Promega Peptide 3 (SEQ
ID NO:3), Promega Peptide 7 (SEQ ID NO:7) and analogs
thereof.

     26.   A bioreagent for quantitating the presence of
tyrosine kinase, which bioreagent is selected from the
group consisting of Promega Peptide 8 (SEQ ID NO:8) and
analogs thereof.

     27.   A bioreagent for quantitating the presence of
protein kinase C, which bioreagent is selected from the
group consisting of Promega Peptide 4 (SEQ ID NO:4),
Promega Peptide 5 (SEQ ID NO:5), Promega Peptide 6 (SEQ
ID NO:6), Promega Peptide 11 (SEQ ID NO:11) and analogs
thereof.

     28.   A bioreagent for quantitating the presence of
proteases, which bioreagent is selected from the group
consisting of Promega Peptide 1 (SEQ ID NO:1), Promega
Peptide 2 (SEQ ID NO:2), Promega Peptide 3 (SEQ ID NO:3),
Promega Peptide 4 (SEQ ID NO:4), Promega Peptide 5 (SEQ
ID NO:5), Promega Peptide 6 (SEQ ID NO:6), Promega
Peptide 7 (SEQ ID NO:7), Promega Peptide 8 (SEQ ID NO:8),
Promega Peptide 9 (SEQ ID NO:9), Promega Peptide 10 (SEQ
ID NO:10), Promega Peptide 11 (SEQ ID NO:11) and analogs
thereof.

     29.   A bioreagent for quantitating the presence of
HIV protease, which bioreagent is selected from the group
consisting of Promega Peptide 9, (SEQ ID NO:9) and
Promega Peptide 10 (SEQ ID NO:10) and analogs thereof.

     30.   A kit for quantitating the presence or activity
of an enzyme comprising:

a.   a container containing a substrate modified peptide having specific reactivity to the enzyme, modified by chemical reaction to allow easy visualization/quantitation; and

b.   instructions for use.

31.   The kit of claim 30 wherein the substrate modified peptide includes a detector segment selected from the group consisting of dansyl, sulforhodamine, lissamine rhodamine (rhodamine B) and fluorescein.

32.   The kit of claim 30 further including a container containing at least one buffer, which buffer is compatible with the enzyme.

33.   The kit of claim 30 further including a container containing at least one separation matrix for isolation of either a product modified peptide or the substrate modified peptide.

34.   The kit of claim 30 wherein the substrate modified peptide is selected from the group consisting of the following: Promega Peptide 1 (SEQ ID NO:1), Promega Peptide 2 (SEQ ID NO:2), Promega Peptide 3 (SEQ ID NO:3), Promega Peptide 4 (SEQ ID NO:4), Promega Peptide 5 (SEQ ID NO:5), Promega Peptide 6 (SEQ ID NO:6), Promega Peptide 7 (SEQ ID NO:7), Promega Peptide 8 (SEQ ID NO:8), Promega Peptide 9 (SEQ ID NO:9), Promega Peptide 10 (SEQ ID NO:10), Promega Peptide 11 (SEQ ID NO:11) and analogs thereof.

35.   The kit of claim 30, wherein the enzyme is selected from the group consisting of kinases, phosphatases and proteases.

36.   A kit for quantitating the presence or activity of c-AMP dependent kinase comprising:

a.   a container containing a substrate modified peptide selected from the group consisting of Promega Peptide 1 (SEQ ID NO:1), Promega Peptide 2 (SEQ ID NO:2), Promega Peptide 3 (SEQ ID NO:3), Promega Peptide 7 (SEQ ID NO:7), analogs and combinations thereof; and

b.   instructions for use.

37.   A kit for quantitating the presence or activity of tyrosine kinase comprising:

    a.   a container containing a substrate modified peptide selected from the group consisting of Promega Peptide 8 (SEQ ID NO:8), analogs and combinations thereof; and

    b.   instructions for use.

38.   A kit for quantitating the presence or activity of protein kinase C comprising:

    a.   a container containing a substrate modified peptide selected from the group consisting of Promega Peptide 4 (SEQ ID NO:4), Promega Peptide 5 (SEQ ID NO:5), Promega Peptide 6 (SEQ ID NO:6), Promega Peptide 11 (SEQ ID NO:11), analogs and combinations thereof; and

    b.   instructions for use.

39.   A kit for quantitating the presence or activity of proteases comprising:

    a.   a container containing a substrate modified peptide selected from the group consisting of Promega Peptide 1 (SEQ ID NO:1), Promega Peptide 2 (SEQ ID NO:2), Promega Peptide 3 (SEQ ID NO:3), Promega Peptide 4 (SEQ ID NO:4), Promega Peptide 5 (SEQ ID NO:5), Promega Peptide 6 (SEQ ID NO:6), Promega Peptide 7 (SEQ ID NO:7), Promega Peptide 8 (SEQ ID NO:8), Promega Peptide 9 (SEQ ID NO:9), Promega Peptide 10 (SEQ ID NO:10), Promega Peptide 11 (SEQ ID NO:11), analogs and combinations thereof; and
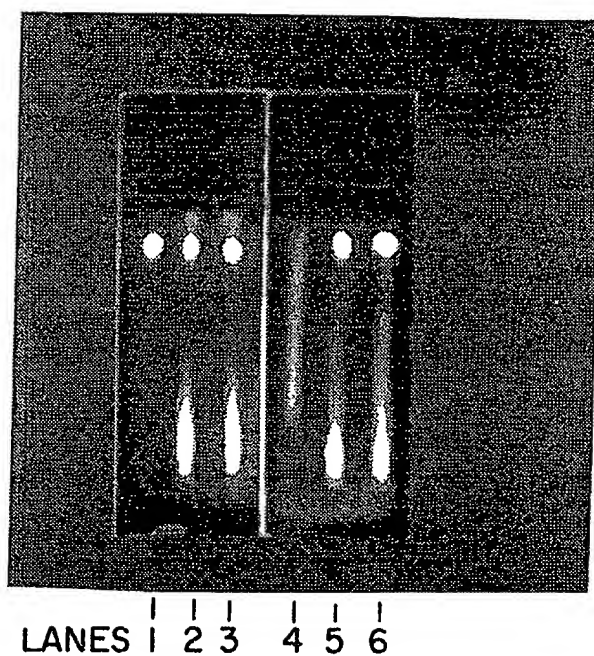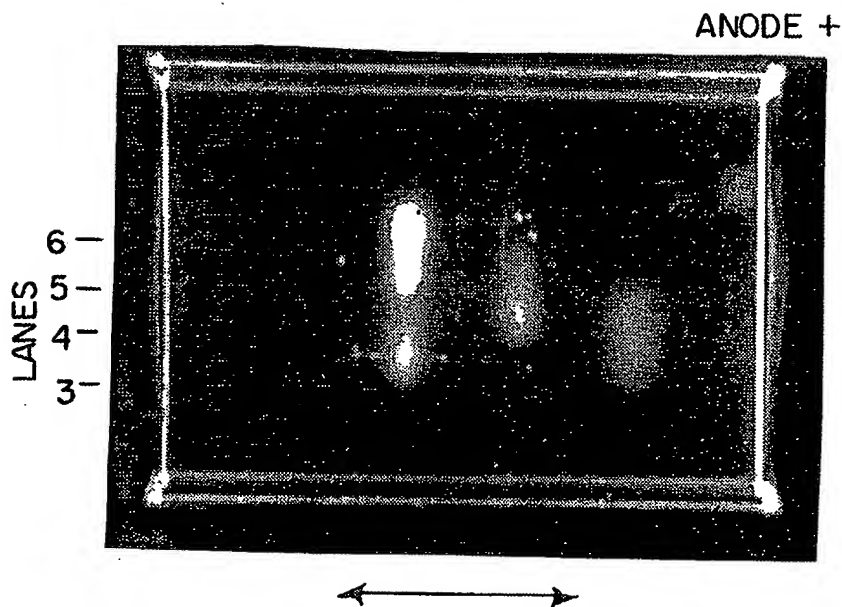
    b.   instructions for use.

40.   A kit for quantitating the presence or activity of HIV protease comprising:

    a.   a container containing a substrate modified peptide selected from the group consisting of Promega Peptide 9 (SEQ ID NO:9), Promega Peptide 10 (SEQ ID NO:10), analogs and combinations thereof; and

    b.   instructions for use.

41.   A kit for quantitating the presence or activity of phosphatases comprising:

a.   a contain r containing a substrate modified peptide selected from the group consisting of Promega Peptide 1 (SEQ ID NO:1), Promega Peptide 2 (SEQ ID NO:2), Promega Peptide 3 (SEQ ID NO:3), Promega Peptide 4 (SEQ ID NO:4), Promega Peptide 5 (SEQ ID NO:5), Promega Peptide 6 (SEQ ID NO:6), Promega Peptide 7 (SEQ ID NO:7), Promega Peptide 8 (SEQ ID NO:8), Promega Peptide 11 (SEQ ID NO:11), analogs and combinations thereof, wherein the substrate modified peptide is derived from the products of the reaction of a protein kinase with the substrate modified peptide; and

b.   instructions for use.

42.   A method for detecting enzymes in a body fluid, comprising reacting the body fluid with a substrate modified peptide under conditions where the enzyme is active for a time sufficient to form a product modified peptide, and measuring the amount of product modified peptide formed.

43.   The method of claim 42 wherein the fluid is blood or lymph fluid.

44.   The method of claim 42 wherein the substrate modified peptide is selected from the group consisting of the following: Promega Peptide 1 (SEQ ID NO:1), Promega Peptide 2 (SEQ ID NO:2), Promega Peptide 3 (SEQ ID NO:3), Promega Peptide 4 (SEQ ID NO:4), Promega Peptide 5 (SEQ ID NO:5), Promega Peptide 6 (SEQ ID NO:6), Promega Peptide 7 (SEQ ID NO:7), Promega Peptide 8 (SEQ ID NO:8), Promega Peptide 9 (SEQ ID NO:9), Promega Peptide 10 (SEQ ID NO:10), Promega Peptide 11 (SEQ ID NO:11) and analogs thereof.

FIG. 1

LANES 1 2 3 4 5 6

ANODE +

LANES 6 5 4 3

FIG.2
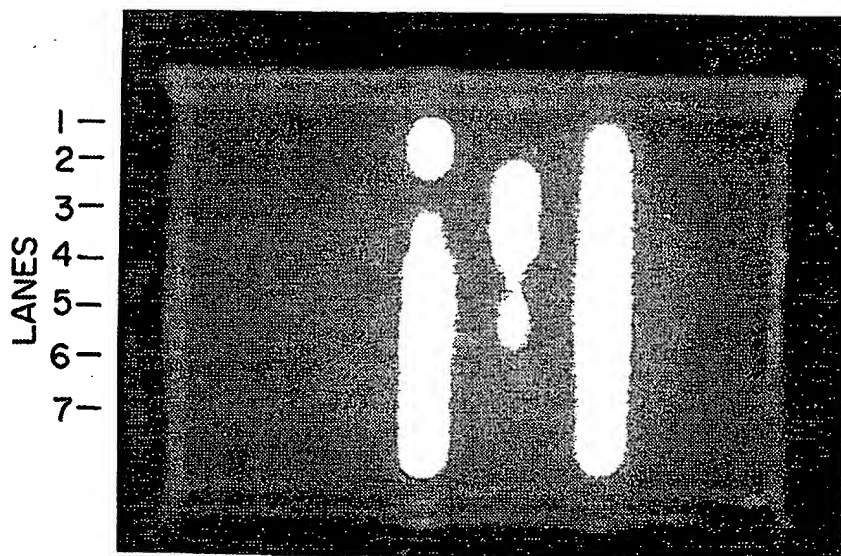
FIG. 3
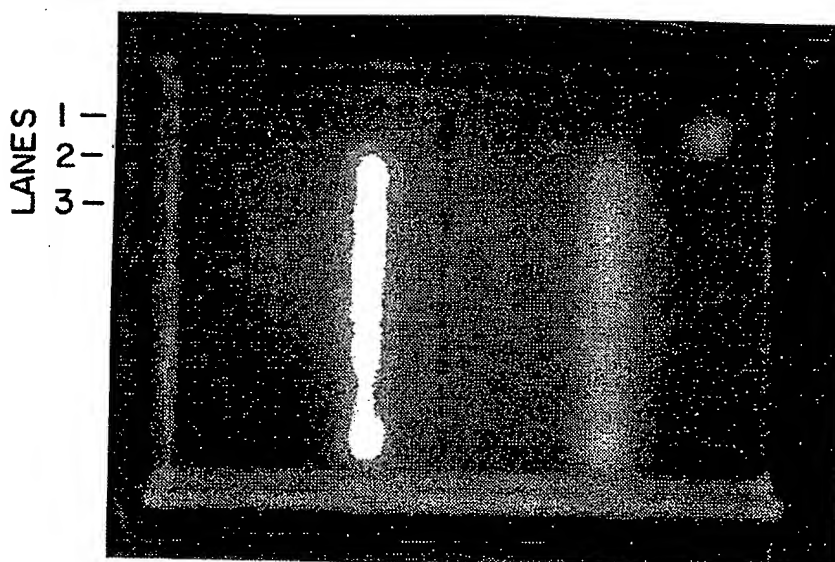
FIG. 4
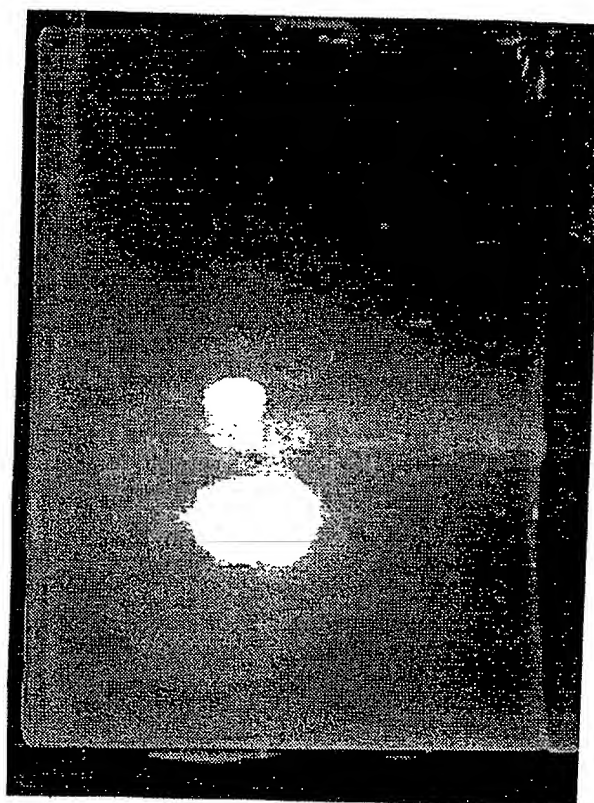
FIG.5

LANES 1 2 3 4

FIG.6
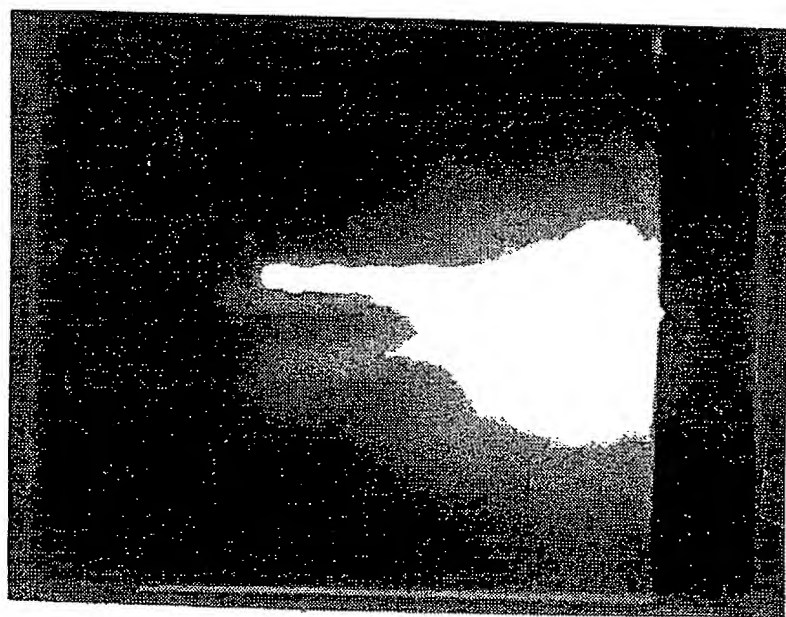
+

LANES 1 2 3 4 5 6 7 8

FIG. 7

LANES
1—
2—
3—
4—
5—
6—
7—
8—
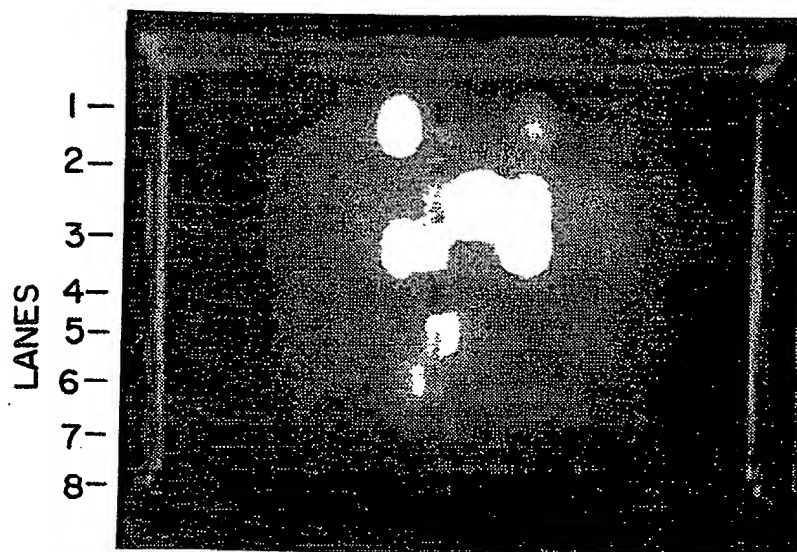
FIG. 8
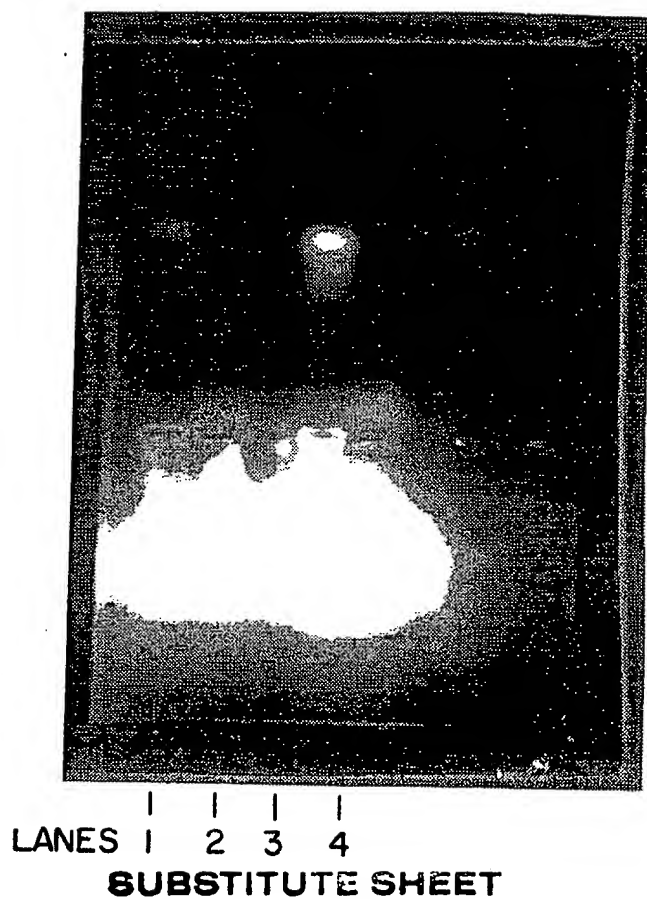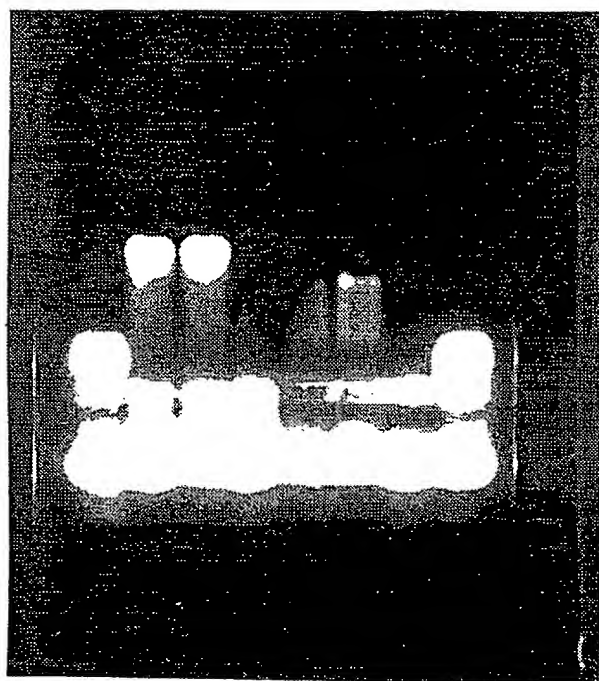
LANES 1   2   3   4

SUBSTITUTE SHEET

FIG.9
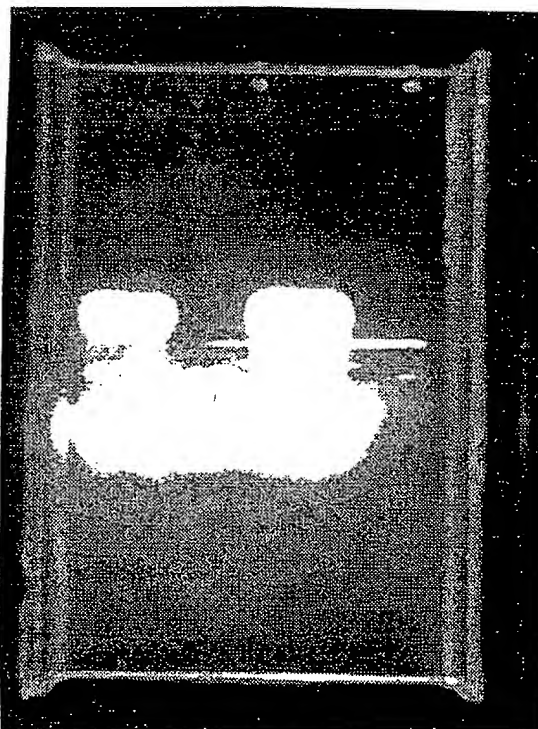
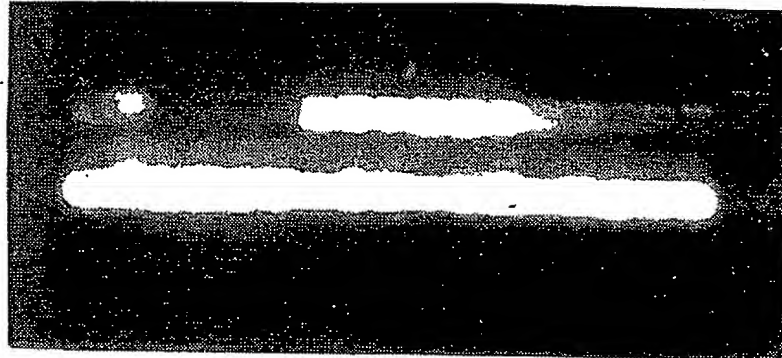LANES 1 2 3 4 5 6 7 8

FIG.10
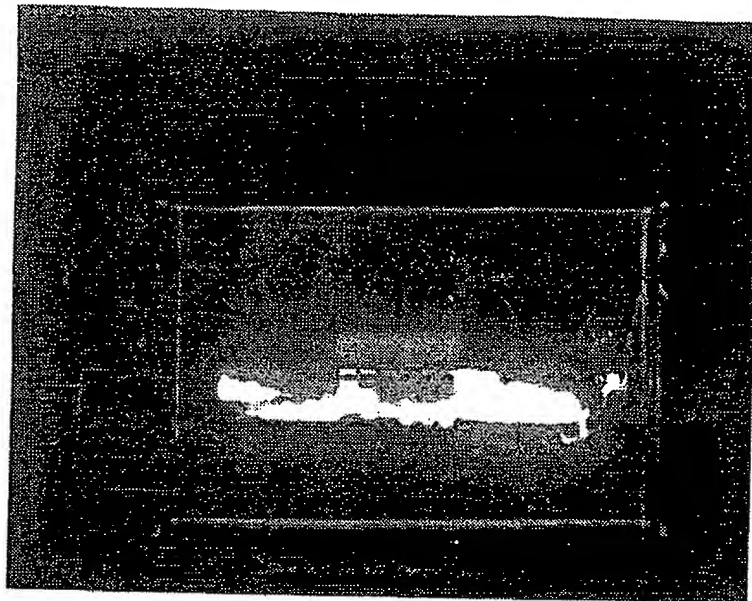
LANES 1 2 3 4 5 6

LANES   1 | 3 | 5 | 7 | 9 | 11 | 13 | 15 | 17 |
         2   4   6   8   10   12   14   16   18

FIG.11

LANES   1 2   3   4 5   6 7   8   9   10 11   12   13 14   15 16 17

FIG.12

FIG.13

LANES 1 2 3 4 5 6 7

FIG.14

LANES 1 2 3 4 5 6 7 8 9 10 11 12 13 14

FIG.15

TOP TIER

BOTTOM TIER

LANES 1 2 3 4 5 6 7

FIG.16

LANES 1 2 3 4 5
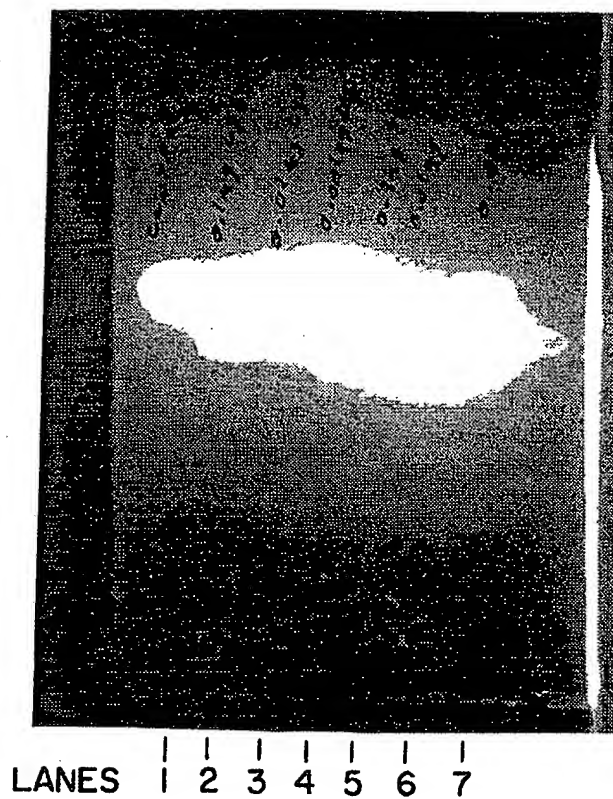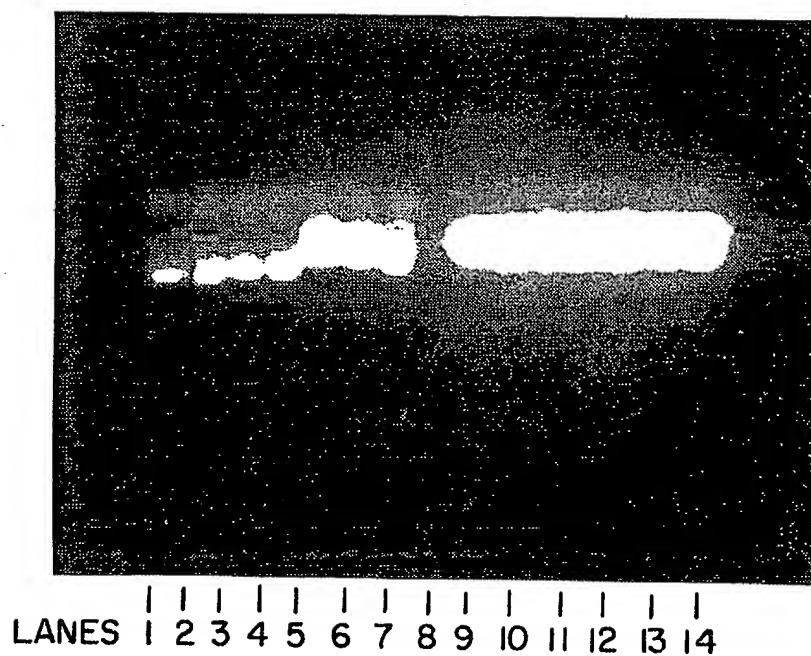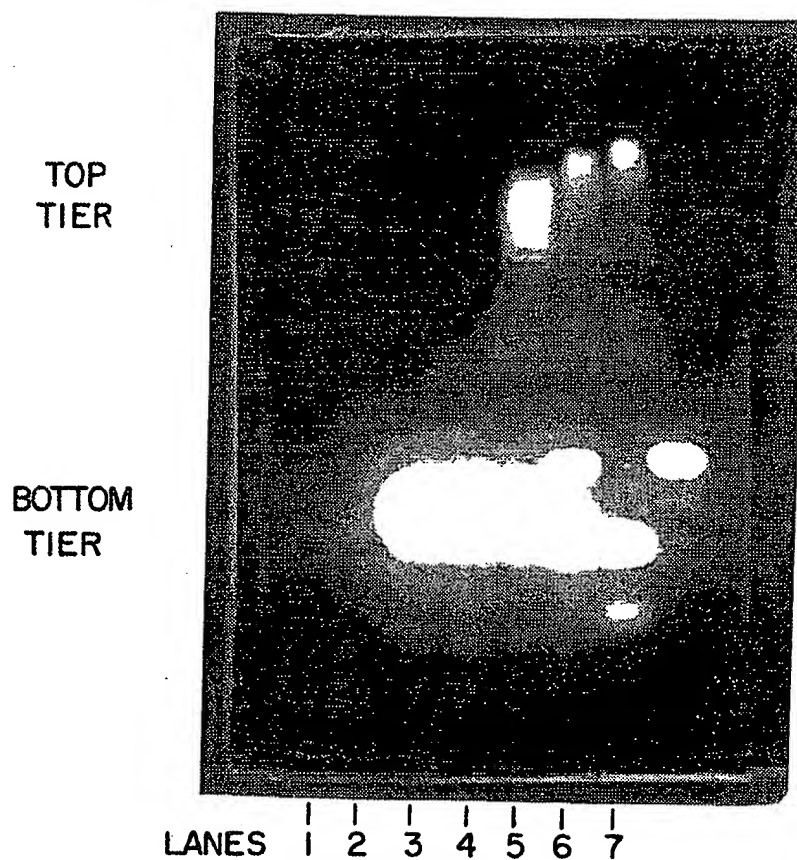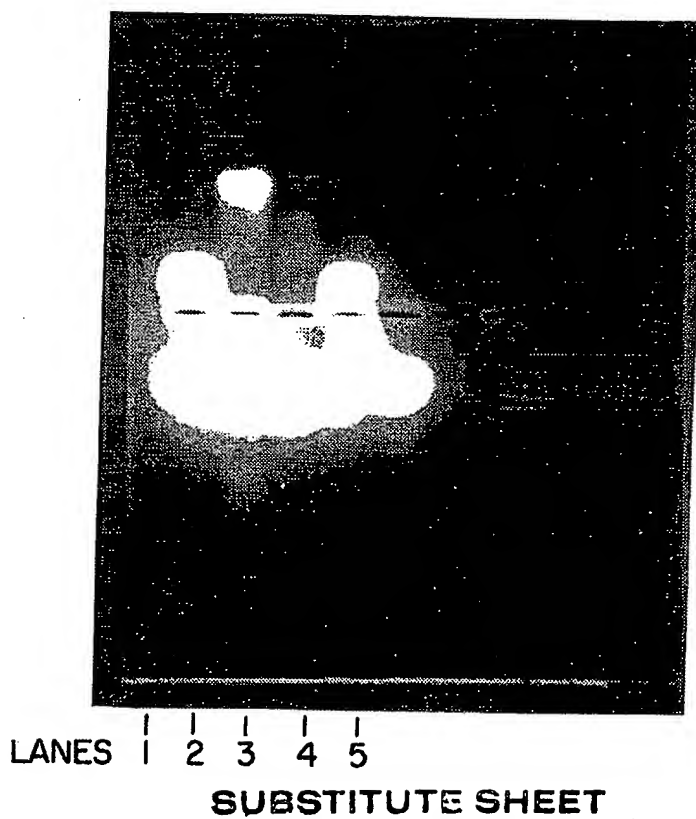
END OF GEL ORIENTED TOWARD
THE POSITIVE ELECTRODE
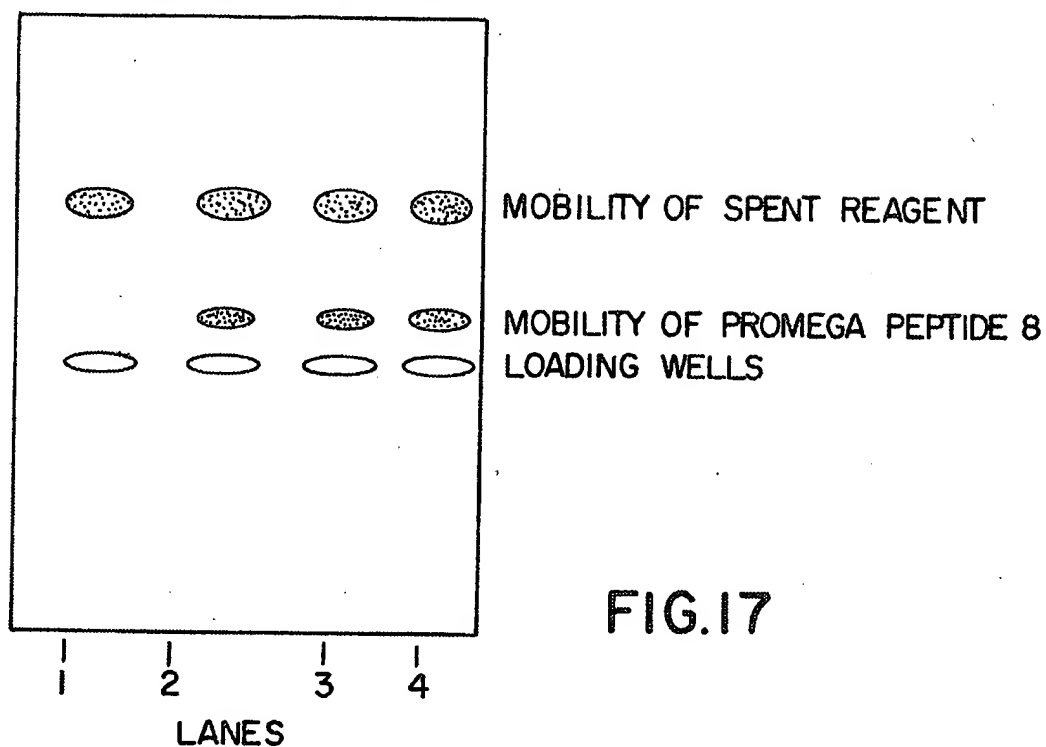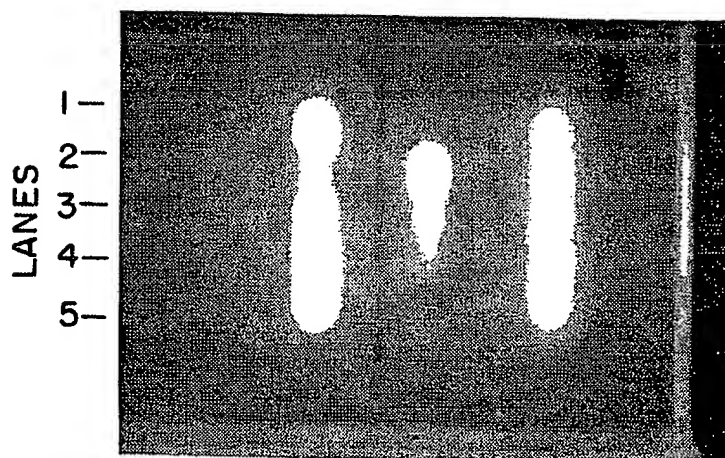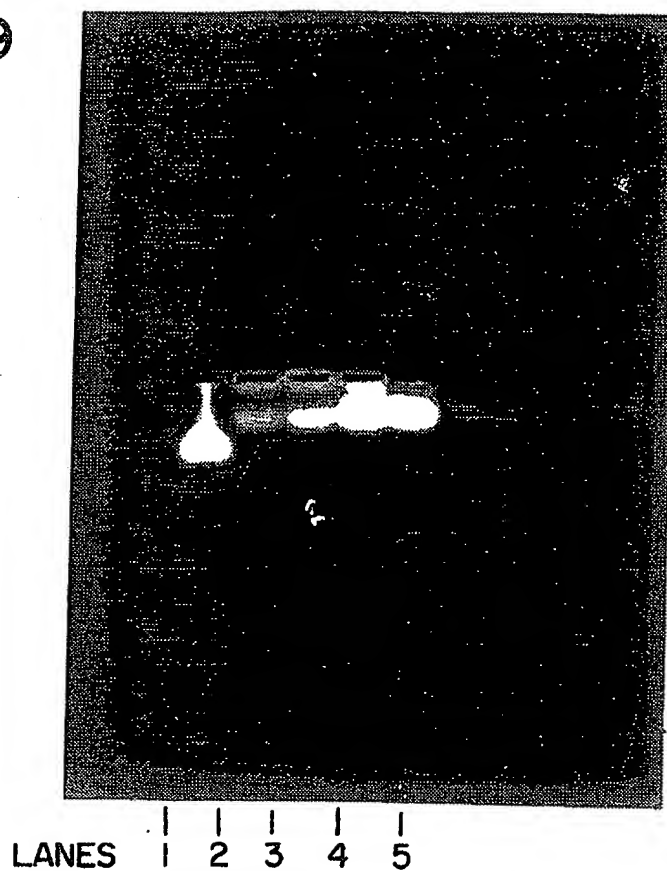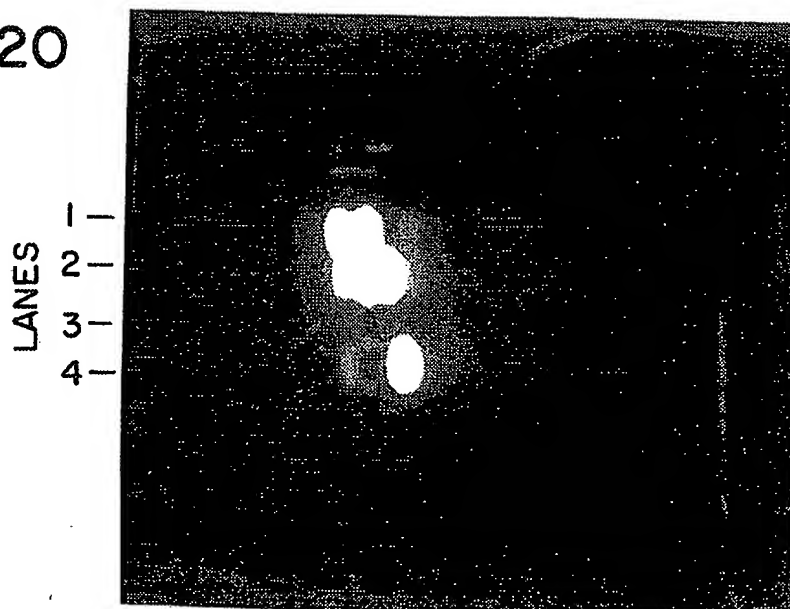
MOBILITY OF SPENT REAGENT

MOBILITY OF PROMEGA PEPTIDE 8
LOADING WELLS

FIG.17

LANES
1 2 3 4

FIG.18

LANES
1—
2—
3—
4—
5—

FIG. 19

LANES   1   2   3   4   5

FIG. 20

LANES   1   2   3   4

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :G01N 33/573; C12Q 1/42; G01N 33/53
US CL :435/24, 7.4, 21, 6, 25, 810, 814, 172.2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/24, 7.4, 21, 6, 25, 810, 814, 172.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS (non-radioactive, peptide substrate, Intell; Genetics (Sequences Promega Peptides 1-11)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 4,681,842 (Rosalki), 21 July 1987, see entire document.	1-44
A	US, A, 4,687,732 (Ward et al), 18 August 1987, see entire document.	1-44
A	US, A, 4,803,157 (Sundberg et al), 07 February 1989, see entire document.	1-44
A	US, A, 4,912,033 (Ladenson et al), 27 March 1990, see entire document.	1-44
Y	Chemical Abstracts, Volume 104(5): 31369X, issued 1985, Dattagupta et al "Nucleic acid-protein conjugate", see entire document.	1-44
Y	Chemical Abstracts, Volume 108(19): 164220w, issued 1988, Kumar et al "Nonradioactive labelling of synthetic oligonucleotide probes with terminal deoxynucleotidyl transferase", see entire document.	1-44
Y	WO, A, 85/00809 (Stammer et al), 28 February 1985, see entire document.	1-44
Y	JP, A, 02,240,027 (AGENCY OF IND SCI TECH), 25 September 1990. See English Abstract and Japanese patent.	1-44

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 DECEMBER 1992	27 JAN 1993
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer EXAMINER LEARY
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages.	Relevant to claim No.
Y	Methods in Enzymology, Volume 200, 1991, Racker, Efraim, "Use of Synthetic Amino Acid Polymers for Assay of Protein-Tyrosine and Protein-Serine Kinases", see pages 107-111.	1-44

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*